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Allosteric interactions between the binding sites of receptor agonists and guanine nucleotides: A comparative study of the 5-hydroxytryptamine$_{1A}$ and adenosine A_1 receptor systems

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Allosteric Interactions Between the Binding Sites of Receptor Agonists and Guanine Nucleotides: A Comparative Study of the 5-Hydroxytryptamine$_{1A}$ and Adenosine A_1 Receptor Systems

by

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The City University of New York

Abstract

Allosteric Interactions Between the Binding Sites of Receptor Agonists and Guanine Nucleotides: A Comparative Study of the 5-Hydroxytryptamine$_{1A}$ and Adenosine A_1 Receptor Systems

by

Cathy D. Mahle

Advisor: Saul Maayani, Ph.D.

Receptor-mediated signal transduction requires interaction between receptor, G-protein, and effector. Agonist binding to receptor stabilizes a high affinity ternary complex, H-R-G. Guanine nucleotides (GN) destablize H-R-G, converting the high affinity into the low affinity state. Characteristics of the allosteric interaction between binding of hormone to receptor and GN to G-protein(s) were investigated.

Initially this interaction was examined in rat hippocampal membranes, using three [^{3}H]-agonists: an AD A_1 agonist ([^{3}H]-R-PIA), and a full ([^{3}H]-8-OH-DPAT) and partial ([^{3}H]-BMY-7378) 5-HT$_{1A}$ agonist. Density (B_{max}, pmol/mg protein) of H-R-G appeared dependent on receptor type and agonist relative efficacy. GN decreased H-R-G density (50%), while K_d values remained constant.

GN attenuated [^{3}H]-agonist binding in a concentration-dependent saturable manner. Parameters of the attenuation (IC_{50}, E_{max} and slope index) were used to characterize the allosterism

occurring between binding of agonist to receptor, and GN to G-protein(s). Similar IC_{50} values were observed for 5-HT_{1A} agonists. Higher IC_{50} values were observed with the AD A_1 agonist at equivalent receptor occupancy. IC_{50} values increased with increasing receptor occupancy; IC_{50} (μM): R-PIA 0.6-2.6; 8-OH-DPAT, BMY-7378 0.18-0.8, indicating IC_{50} values were effected by degree of agonist occupancy, receptor type, and not drug relative efficacy. GTP-dependent inhibition of adenylyl cyclase was indistinguishable across agonists (EC_{50} 0.3-0.5 μM). Events related to drug relative efficacy may occur at R/G interaction and not GN/G-protein or G-protein/effector.

Quantitative receptor autoradiography (QRA) was applied to study this allosterism in rat and human hippocampal subregions. Agonist affinity was similar in hippocampal membranes and subregions. Parameters of GN concentration-effect curves were determined. In contrast to membrane preparations, attenuation of [^{3}H]-agonist binding by Gpp(NH)p in hippocampal subregions was nearly complete. Within regions, IC_{50} values were indistinguishable, but for [^{3}H]-8-OH-DPAT were lower (0.1-0.2 μM) in human than rat (0.3-0.4 μM). In human hippocampus the IC_{50} of the GN curves was lower for [^{3}H]-8-OH-DPAT than [^{3}H]-R-PIA (0.2-0.3 μM). Allosteric interactions between binding of hormone and GN to the receptor system can be measured using QRA, enabling discrete localization and characterization of H-R-G.

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FORMAT OF Ph.D DISSERTATION

This dissertation is prepared according to the new guidelines of the City University of New York. These guidelines permit direct incorporation of research articles to be submitted for publication as chapters in the thesis. The overall format contains an abstract, table of contents, general introduction, three chapters, a general discussion, and bibliography. Each individual chapter contains an abstract, an introduction, materials and methods, results, discussion, and tables and figures.

ABBREVIATIONS

5-HT - 5-hydroxytryptamine

8-OH-DPAT - 8-hydroxy-2-(di-n-propylamino)tetralin

AD A_1 - adenosine A_1

ANOVA - analysis of variance

B_{max} - maximal number of binding sites

CPT - 8-cyclopentyl-1,3-dimethylxanthine

DPCPX - 8-cyclopentyl-1,3-dipropylxanthine

EGTA - ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EC_{50} - midpoint in the concentration-effect curve

E_{max} - maximal effect

G-protein(s) - guanine nucleotide regulatory binding protein(s)

GN - guanine nucleotides

Gpp(NH)p - guanosine 5'-(β, γ-imido)triphosphate

GTPγS - guanosine 5'-O-(3-thiophosphate)

H-R-G - hormone / receptor / G-protein complex

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

K_d - agonist dissociation constant

K_H -agonist dissociation constant of the high affinity state of the receptor

K_L -agonist dissociation constant of the low affinity state of the receptor

n - slope index

n_H - Hill coefficient

PAA - 2-phenylaminoadenosine (CV-1808)

R-PIA - R-phenylisopropyladenosine

INTRODUCTION

The Receptor System. The initial steps of signal transduction in excitable cells require sequential interaction between at least three proteins embedded in the cell membrane which constitute the receptor system: **hormone receptor, guanine nucleotide binding protein** (G-protein), and **effector** (an enzyme or channel). Neurotransmitters, hormones, and other endogenous substances bind to distinct receptors, and alter cellular function through generation of intracellular second messengers. Many receptors regulate intracellular effectors through activation of G-proteins, an example being the β-adrenoreceptor which contains seven membrane spanning regions (Lefkowitz and Caron, 1988). The receptor interacts with the **G-protein** (s), that bind and hydrolyze GTP. The G-protein family contains a large number of closely related members, all of which are heterotrimers, consisting of the subunits (in order of decreasing molecular size) α, β, γ (Casey and Gilman, 1988). The α subunit has a single high affinity binding site for GTP and it posses an intrinsic GTPase activity. The α subunit is the site of action of bacterial toxins (cholera and pertussis). The various α-subunits are quite diverse in structure, while the β/γ are more strictly conserved.

A simplistic scheme of the interactions occurring between the components of the receptor system is illustrated in Fig. 1. An assumption is that this scheme is applicable for the two G_i-linked receptor systems selected for this study, the 5-HT_{1A} and the AD A_1

receptor. Based upon their association with G-protein(s) in the solubilized preparation, at least a portion of these receptors are assumed to be pre-coupled to G-proteins (Stiles, 1988; El Mestikawy et al., 1988), as has been reported for the alpha$_2$-adrenergic receptor, also known to act via inhibitory G-proteins (Jagadeesh et al., 1990; Neubig, et al., 1988). Indeed, the ternary complex model proposed by De Lean et al (De Lean, et al., 1980) allows for a portion of the membrane receptors to be "precoupled" to the G-protein(s). Receptors which were uncoupled from G-proteins, or those which are coupled to G-protein with guanine nucleotide bound, display a **low affinity** for the hormone (K_L). The basal rate of GDP dissociation is very slow, and the basal GTPase activity much larger than the rate of nucleotide exchange, therefore, most G-protein exists bound with GDP (Gilman, 1987). Therefore, the finding that only the low affinity state can be measured in intact cells (Gerwins et al., 1990b; Martens et al., 1988) comes as no surprise. Binding a hormone to the receptor activates it (Taylor, 1990a) and catalyzes an exchange between the bound GDP and free GTP (Gilman, 1987). If there is only one GTP/GDP binding site on the α subunit, i.e., if the binding of GDP and of GTP is mutually exclusive, then for a brief time a ternary complex is formed (H-R-G) with an "empty" G-protein. In this form the receptor possess **high affinity** for the hormone (K_H). (De Lean et al., 1980). GTP has a high affinity for the empty G-protein to which it binds, and promotes dissociation of the α from the $\beta\gamma$ subunit, consequently destabilizing H-R-G. The destabilization of the ternary complex converts the receptor system back to the low affinity state. The activated α-GTP interacts with an **effector system.** GTP is then

hydrolyzed to GDP, allowing recombination of α-GDP with βγ, and inactivation of the cycle. Non-hydrolyzable analogs of GTP, (e.g., GTPγS or Gpp(NH)p) cause a persistent activation of the α subunit.

The various intermediates can be directly or indirectly probed in the laboratory. *In vitro*, guanine nucleotides can be essentially removed from a membrane preparation, by extensive washing in high concentrations of Mg^{++}; and restriction of [^{3}H]-agonist concentration, so only high affinity binding is measured. Binding of guanine nucleotides to the G-protein causes destabilization of H-R-G (De Lean, et al., 1980), and the loss of high affinity binding is assumed to be due to conversion to the low affinity state. As antagonists competition curves yield slope indexes of unity and are unaffected by guanine nucleotides (Kent et al., 1980; Shearman and Strange, 1988), it has been suggested that they do not differentiate between affinity states. This should result in larger B_{max} values observed in a given system with labeled antagonists, as compared to agonists. This phenomenon has been shown to exist in membrane preparations for several receptor systems, including the 5-HT_2 (Lyon et al., 1987), α-adrenergic (Neubig et al., 1985; Galitzky et al., 1989), and the AD A_1 (unpublished observation).

Agonist binding to sites labeled by antagonist, generates curves with slope indexes significantly less that unity (Lefkowitz, et al., 1976b; Shearman and Strange, 1988), that can be deconvoluted into high and low affinity components (De Lean et al., 1980; Kent et al., 1980). Addition of guanine nucleotides 'steepens' the slope of the

curves and converts the majority of sites into the low affinity state. The low affinity state is the result of GTP-destabilization of H-R-G, and the subsequent α-GTP activation of an effector, thus it has been hypothesized as the affinity state mediating the functional response, in membrane preparations (Ehlert 1985; Jakobs et al., 1978), and intact cells (Nathanson, 1983; Ströher et al., 1989).

Adenosine A_1 Receptor. Adenosine is present in the CNS as the result of its role as a metabolic intermediate or from *de novo* biosynthesis (Snyder, 1985). Indeed, ATP is packed, stored, and co-released with other neurotransmitters (Richardson et al., 1987; Pelleg and Burnstock, 1990). The clinical relevance of adenosine receptors has been inferred due to its role as an anticonvulsant (Dragunow and Faull, 1988), vasodilator (Sabouni et al., 1990), and anxiolytic (Phillis and O'Regan, 1988). Adenosine may also play an important role in psychiatric disorders (Durcan and Morgan, 1990), and in the ischemia following a stroke (Januszewicz et al., 1989; Daval et al., 1989b).

Adenosine actions in the CNS are mediated by at least two receptors subtypes: AD A_1 and AD A_2 (Van Calker et al., 1979), whose actions are inhibited by methylxanthine phosphodiesterase inhibitors, caffeine and theophylline (Sattin and Rall, 1970). The AD A_2 receptor is discretely localized within the CNS, to the striatum and olfactory tubercle (Jarvis and Williams, 1989). In contrast, the AD A_1 receptor, which displays heterogeneity with regard to density within the CNS, is ubiquitously distributed (Fastbom et al., 1987a; Fastbom et al., 1987b). The AD A_1 receptor can be radiolabeled by the

agonists [^{3}H]-cyclohexyladenosine (Goodman and Snyder, 1982), [^{3}H]-R-Phenylisopropyladenosine ([^{3}H]-R-PIA, Schwabe and Trost, 1980), and the antagonist [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-DPCPX, Lohse, et al., 1987). [^{3}H]-R-PIA is highly specific, is very high affinity, and is available with the active stereoisomer labeled (Schwabe and Trost, 1980). Functionally, the AD A_1 receptor inhibits the production of cAMP, by the inhibition of adenylyl cyclase (Ebersolt et al., 1983), and mediates the hyperpolarization of cells, by the opening of potassium channels (Trussell and Jackson, 1987). While numerous groups report success on solubilization of the AD A1 receptor (Gavish et al., 1982; Klotz et al., 1986; Helmke and Cooper, 1989; Casadó et al., 1990; Olah et al., 1990), this receptor has yet to be sequenced and cloned. Solubilization has revealed that the AD A_1 receptor is associated with a G-protein(s) (Stiles, 1988).

Serotonin$_{1A}$ receptor. Serotonin is a neurotransmitter whose actions have been linked to appetite (Leibowitz, 1990), memory (Hunter, 1989), hallucinogenic behavior (Titeler et al., 1988), migraine (Humphrey et al., 1990), sexual behavior (Mendelson and Gorzalka, 1986), cardiovascular regulation (Mandal et al., 1989, Kolassa et al., 1989), and anxiety and depression (Charney et al., 1990; Taylor, 1990b). These physiological responses are no doubt mediated by one or more of the 5-HT receptors known to exist, including 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 receptors. The 5-$HT_{1\text{-like}}$ receptors, which display a high affinity for [^{3}H]-5-HT, can be further subdivided into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1P}, 5-HT_{1E}.

The 5-HT_{1A} receptor, which has been implicated in many of the above physiological processes, is less ubiquitously distributed than the AD A_1 receptor, and is found in highest concentrations in the raphe nuclei and hippocampus of both rodent (Pazos and Palacios, 1985) and human (Pazos et al., 1987a; Hoyer et al., 1986) brain. Under proper conditions, this receptor can be labeled using [^{3}H]-5-HT (Pazos and Palacios, 1985), [^{3}H]-WB-4101 (Norman et al., 1985), [^{3}H]-ipsapirone (Glaser et al., 1985), and [^{3}H]-spiroxatrine (Nelson et al., 1987). The most selective [^{3}H]-agonist available is (±)-[^{3}H]-8-OH-DPAT (Gozlan et al., 1983). This radioligand is highly stable, and possesses a specific activity 5-6 times greater than that of [^{3}H]-5-HT. Also, due to its high specificity, no masking of the other subtypes of 5-HT_1 receptors is necessary, as with [^{3}H]-5-HT. Despite the numerous [^{3}H]-agonists available for radioligand binding, currently no selective, high affinity antagonist is known.

The 5-HT_{1A} receptor subtype has been found to be functionally linked to two effector systems: 1). inhibition of adenylyl cyclase (De Vivo and Maayani, 1986), and 2). membrane hyperpolarization, due to the opening of potassium channels (Andrade et al., 1986). The 5-HT_{1A} receptor has been solubilized (Asarch and Shih, 1987; El Mestikawy et al., 1988), and is associated with a G-protein(s) (Emerit et al., 1990; Kline et al., 1989). The human 5-HT_{1A} receptor gene has been cloned and the receptor expressed (Fargin et al., 1988; Fargin et al., 1989; Raymond et al., 1989).

Background. 5-HT_{1A} and AD A_1 receptors are distributed in a very high density in both rat and human hippocampus (Fig. 2,3, and results CHAPTERS 1-3). These receptors are also linked to similar effector mechanisms, i.e., inhibition of forskolin-stimulated adenylyl cyclase, and opening of postassium channels. Considering these similarities, as well as the non-additivity in the maximal responses (inhibition of forskolin-stimulated adenylyl cyclase or the amount of outward current evoked) of these receptors, and the fact that *in vivo* treatment of rats with pertussis toxin, caused a similar decrease in the maximal response, Zgombick et al. (Zgombick et al., 1989) proposed that these two receptors may share a common pool of G-proteins.

In order to fully characterize the interaction occurring between the hormone receptor, and the G-protein(s), it is necessary to consider the allosteric interaction which occurs between hormone binding to the receptor, and guanine nucleotide binding to G-proteins. I have chosen to examine this phenomenon in membrane preparations for two receptors which may share a common pool of G-proteins, and exist in high densities in a common brain region. This was done in an effort to examine these allosteric interactions as they differ between receptor systems, species, region, and hormone efficacy.

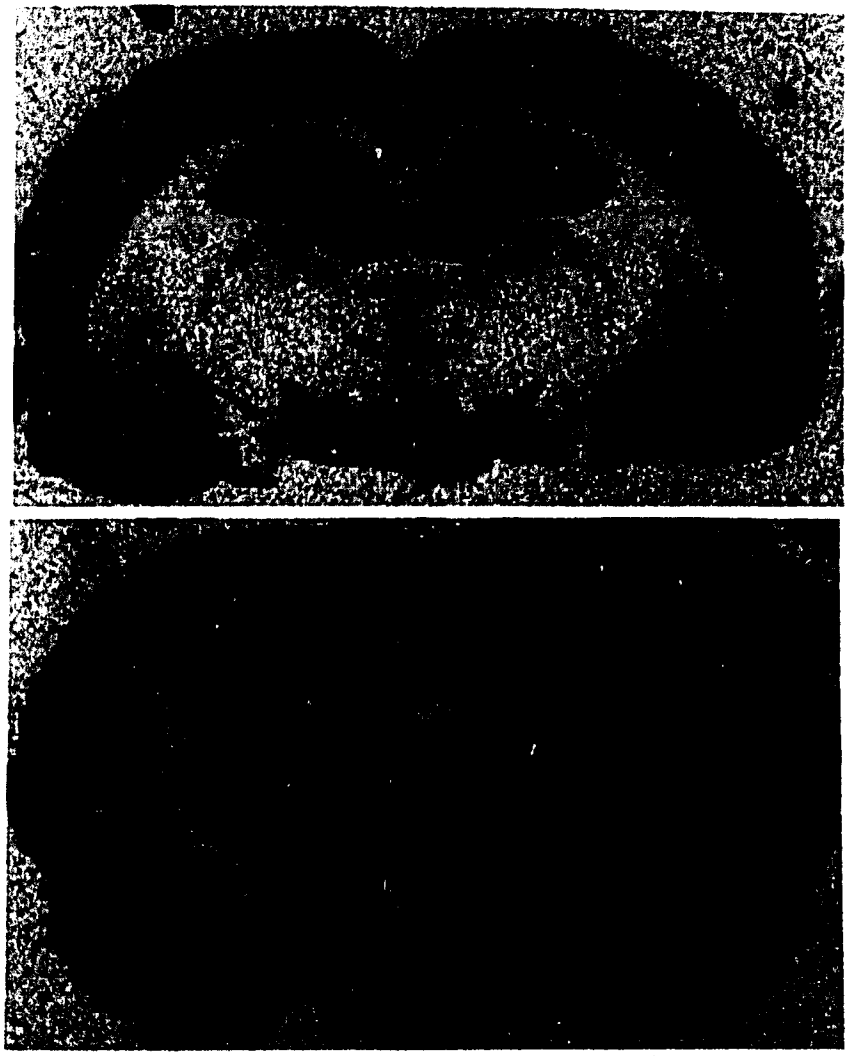
Membrane homogenates have yielded much knowledge concerning receptor system interactions. Using this preparation, one lacks the ability to discretely localize and probe the initiation of

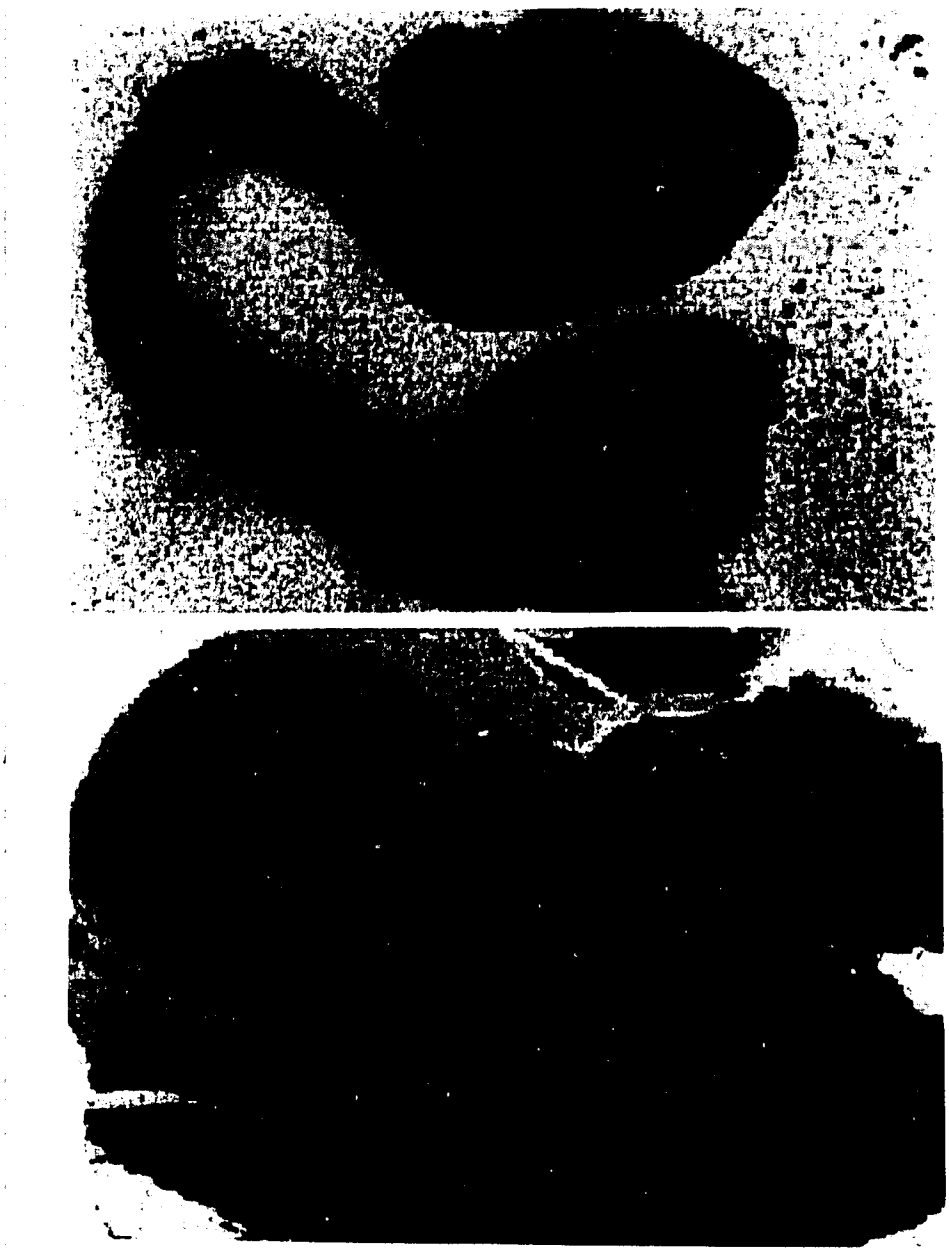
receptor-mediated signal transduction in subregions of a tissue. The introduction of quantitative receptor autoradiography to study neurotransmitter receptors (Kuhar et al., 1986), has yielded invaluable knowledge concerning receptor distribution patterns. This technique provides discrete localization of receptor binding sites, and can be used to evaluate binding parameters as well as pharmacology. This technique can be taken further and used to evaluate the interaction occurring between hormone, receptor and G-protein (CHAPTER 3) and affords the luxury of localization to very small regions. Through this study, an important step has been taken toward the ultimate integration of cellular communication within the central nervous system.

Fig. 1. Hormonal activation of the initial stages of signal transduction mediated by G_i-linked receptors. A full description of the G-protein cycle is described in the text.

H
Adenylyl Cyclase
R_L
α_i
GDP
$\beta\gamma$
Adenylyl Cyclase
H
R_L
$\beta\gamma$
GTP
GDP
α_i
ATP
Adenylyl Cyclase
cAMP
R_H
H
GDP
α_i
GTP
$\beta\gamma$
Adenylyl Cyclase
GTP
α_i
R_L
$\beta\gamma$
H

Figs. 2 and 3. (±)-[^{3}H]-8-OH-DPAT (top) and [^{3}H]-R-PIA (bottom) to 15 μ sections of rat (Fig. 2) brain, and human hippocampus (Fig. 3). The in vitro binding was performed as described in CHAPTER 2, Materials and Methods.





Allosteric Interactions Between the Binding Sites of Receptor Agonists and Guanine Nucleotides: A Comparative Study of the 5-Hydroxytryptamine$_{1A}$ and Adenosine A$_1$ Receptor Systems in Rat Hippocampal Membranes

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ABSTRACT

The high affinity ternary complex formed in a cell free system between a hormone or agonist, a receptor and a guanine nucleotide binding protein (G-protein), and its destabilization by guanine nucleotides can be utilized as a model to study early events involved in the inititiation of signal transduction. In the present study, we have characterized the allosteric interactions between the binding of agonist and guanine nucleotides to the receptor/G-protein complex. Two inhibitory G-protein linked receptors, the adenosine A_1, and 5-hydroxytryptamine$_{1A}$ receptors in rat hippocampal membranes were utilized as a model.

The functional interaction between the ternary complex and a guanine nucleotide (GTP) was examined in this cell free system by assay of one of the common effector systems linked to these receptors, inhibition of adenylyl cyclase activity. Concentration-effect curves to GTP were constructed in the presence of a saturating concentration (10 μM) of each of the tested drugs. The GTP- and agonist-dependent inhibition of forskolin-stimulated adenylyl cyclase activity showed similar EC_{50} values for GTP (0.6-2.6 μM) for two adenosine A_1 and four 5-hydroxytryptamine$_{1A}$ agonists regardless of drug relative efficacy. Similarly, the slope indices of all curves (0.8-1.4) were not different from unity. The observed E_{max} values were consistent with reported drug relative efficacy.

The binding of a full adenosine A_1 agonist ([^{3}H]-R-PIA), as well as a full ((±)-[^{3}H]-8-OH-DPAT) and partial ([^{3}H]-BMY-7378) 5-hydroxytryptamine$_{1A}$ agonist were examined in relation to the binding of Gpp(NH)p and GTPγS. The amount of ternary complex formed (B_{max}; pmol/mg), was dependent upon both receptor type and drug relative efficacy: 1.44 ± 0.16 ([^{3}H]-R-PIA), 1.04 ± 0.03 ((±)-[^{3}H]-8-OH-DPAT) and 0.65 ±0.08 ([^{3}H]-BMY-7378). Similarly, the ratio between the drug's EC_{50} value (adenylyl cyclase assay) and dissociation constant (K_d, binding assay) was also receptor type and drug efficacy dependent: 160 (R-PIA), 72((±)-8-OH-DPAT) and 10 (BMY-7378). Destabilization of the ternary complex by 100 μM Gpp(NH)p caused an approximately 50% decrease in the B_{max} for all drugs with no detectable change in the K_d values.

Progressive occupation of the G-protein with increasing concentrations (0.1 nM - 100 μM) of either Gpp(NH)p or GTPγS resulted in a concentration-dependent and saturable attenuation of [^{3}H]-agonist binding. Parameters of the concentration-effect curves (EC_{50}, slope index and E_{max}) were determined for these guanine nucleotides at six different initial levels of receptor occupancy by the [^{3}H]-agonists (24-93% occupancy). For all [^{3}H]-agonists, the EC_{50} values for guanine nucleotide attenuation of binding increased two to six fold with increasing initial receptor occupancy. Both guanine nucleotides exhibited approximately one tenth lower EC_{50} values for the attenuation of [^{3}H]-agonist binding to the 5-hydroxytryptamine$_{1A}$ receptor than for the adenosine A_1 receptor. Similar EC_{50} values were obtained for inhibition of (±)-[^{3}H]-8-OH-

DPAT and [^{3}H]-BMY 7378 binding, suggesting that this effect on receptor/G-protein coupling is not dependent on agonist efficacy.

We propose that stabilization of the ternary complex by hormone binding, as measured by the B_{max} value, is directly related to drug efficacy. Consequently, the amount of ternary complex available for destabilization by guanine nucleotides is greater for the more efficacious agonist. This is then observed as a greater efficacy in the maximal inhibition of forskolin-stimulated adenylyl cyclase. In this system, efficacy appears to be associated with hormone binding to and causing stabilization of a high affinity ternary complex, which constitute the earliest stages of signal transduction.

INTRODUCTION

Binding of a hormone to a membrane receptor initiates a series of membrane and intracellular events collectively referred to as signal transduction (for reviews see Gilman, 1987; Stryer, 1986; Taylor, 1990; Birnbaumer, 1990). Many receptors are linked to guanine nucleotide binding regulatory proteins (G-proteins) which in turn cause activation of an effector(s) (Casey and Gilman, 1988). A thoroughly studied hormone/receptor system is the beta adrenergic receptor (BAR), which activates the enzyme adenylyl cyclase, through coupling to a stimulatory G-protein, G_s (Lefkowitz et al., 1976a). DeLean *et al.* have proposed a model for the BAR, in which the preformed receptor/G-protein complex (R-G) is stabilized by the binding of agonist or hormone (H) to form a ternary complex, H-R-G (De Lean et al., 1980). Binding of a guanine nucleotide to the α subunit of the G-protein, destabilizes this complex leaving the α–guanine nucleotide subunit free to interact with the effector. The H-R complex exhibits a low affinity for the hormone which accelerates its dissociation. Thus, binding of guanine nucleotide to the H-R-G complex allosterically affects agonist binding to the receptor, through interconversion of a high affinity state into a low affinity state of the H-R-G complex (De Lean et al., 1980).

There is considerable evidence consonant with the concept of multiple hormone-sensitive interconvertible receptor affinity states. For example, concentration-response curves of hormone (agonist) competition for a homogeneous population of antagonist binding sites

displays slope indices that are significantly less than unity, e.g., the beta adrenergic (Lefkowitz et al., 1976b), 5-hydroxytryptamine$_2$ (5-HT) (Leysen et al., 1982; Shearman and Strange, 1988), dopamine D_1 (Andersen and Jansen, 1990), and dopamine D_2 (Ohara et al., 1988). By assuming a non-interacting-two affinity state model, deconvolution of these complex curves produced affinity and density parameters relating to both a high and low affinity state (De Lean et al., 1980; Kent et al., 1980; Andersen and Jansen, 1990; Ohara et al., 1988). In most cases, the conversion of the high affinity state to the low affinity state could be achieved by the addition of guanine nucleotides, where a dextral shift in the agonist binding curves and an increase of receptors in the low affinity state was observed. In contrast, binding of an antagonist to the same receptor sites, measured either as direct [^{3}H]-antagonist binding or as binding competition, produces a homogenous population of sites, as reflected by slope indices not significantly different from unity. Moreover, addition of guanine nucleotides do not alter any of the binding parameters (Kent et al., 1980, Shearman and Strange, 1988). Based upon these sorts of experiments, it has been suggested that antagonists do not differentiate between the high and low affinity states of receptors (Lefkowitz et al., 1976b). Consequently, it is expected that under the same experimental conditions, for a homogenous population of binding sites, that an antagonist should label a greater density of binding sites than an agonist radioligand. This phenomenon has been shown to exist in membrane preparations for several receptor systems, including the 5-HT$_2$ (Lyon et al., 1987), alpha adrenergic (Neubig et al., 1985; Galitzky et al., 1989), and

adenosine (AD) A_1 (unpublished observation). Recently, using the agonist [^{3}H]-4-bromo-2,5-diethoxyphenlyisopropylamine (DOB) and the antagonist [^{3}H]-ketanserin, this phenomenon has been shown to exist in clonal cell lines transfected with and expressing the rat (Teitler et al., 1990) and the human (Branchek et al., 1990) 5-HT_2 receptor. This supports the hypothesis that by restriction of [^{3}H]-agonist concentration to the nM range, agonists bind mainly to the high affinity state, while antagonists do not differentiate between affinity states.

AD A_1 and 5-HT_{1A} receptors are coupled to adenylyl cyclase through inhibitory G-proteins (G_i, Ebersolt et al., 1983; Fredholm et al., 1986; De Vivo and Maayani, 1986; Zgombick et al., 1989). Previously, we have postulated that these receptors are colocalized on the same hippocampal pyramidal cells and may share a common population of G-proteins (Zgombick et al., 1989). Agonist binding to both AD A_1 and 5-HT_{1A} receptors is modulated by guanine nucleotide (Bruns et al., 1980; Yeung and Green, 1984; Lohse et al., 1984; Gozlan et al., 1983; Schlegel and Peroutka, 1986; Harrington et al., 1988). Similar to the ternary complex model proposed for the G_s-linked BAR, this modulation may reflect conversion of the high affinity state to the low affinity state. Using the AD A_1 antagonist, [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine (DPCPX), Ströher *et al.* have shown that agonist competition produced shallow displacement curves, consistent with agonist related affinity states (Ströher et al., 1989). As in the G_s-linked BAR, addition of guanine nucleotide caused a dextral shift in the agonist displacement curves, coupled

with an increase in the slope index, which resulted in a larger proportion of receptors in the low affinity state.

The lack of a specific antagonist radioligand for the 5-HT_{1A} receptor has prevented similar analysis of affinity states. Numerous attempts to synthesize a specific antagonist for the 5-HT_{1A} receptor resulted in ligands which display partial agonist properties (Nelson et al., 1987; Herrick-Davis and Titeler, 1988; Glennon et al., 1988; Rydelek et al., 1990; Yocca et al., 1987). Based upon [^{3}H]-5-HT_{1A} agonist sensitivity to guanine nucleotides, it can be inferred that the 5-HT_{1A} receptor exists in multiple affinity states.

For the G_s-linked BAR system, Lefkowitz et al. (Lefkowitz et al., 1976b) suggested that the magnitude of the dextral shift of the agonist displacement curve induced by guanine nucleotide, is related to the intrinsic activity of the agonist as measured functionally using the adenylyl cyclase assay. Furthermore, the ability of an agonist to activate adenylyl cyclase correlates with the amount of high affinity ternary complex (H-R-G) formed in the presence of agonist, and with the ratio of dissociation constants of the agonist for the high and low affinity states of the receptor (Kent et al., 1980).

We have tested the applicability of the high affinity ternary complex model to describe two distinct receptors, the AD A_1 and 5-HT_{1A}, which couple to adenylyl cyclase through an inhibitory G-protein. Using high affinity agonist binding with the radioligands [^{3}H]-R-PIA, an AD A_1 agonist, and [^{3}H]-(±)-8-hydroxydipropyl-

aminotetralin ((±)-8-OH-DPAT) and [^{3}H]-(8-[2-[4-(2-methoxy-phenyl)-1-piperazinyl]ethyl]-8-azaspirol[4.5]-decane-7,9-dione dihydrochloride (BMY-7378), both agonists at the 5-HT_{1A} receptor (Cornfield et al., 1991; Yocca et al., 1987), combined with the functional assay, inhibition of forskolin-stimulated adenylyl cyclase, we have examined the formation and destabilization of the ternary complex as a function of receptor type and agonist intrinsic activity.

MATERIALS AND METHODS

Tissue Preparation. For radioligand binding assays, male Sprague Dawley rats (200-250 g, Charles River, Boston, MA) were asphyxiated using CO_2, and decapitated. Hippocampi were dissected on ice, frozen on dry ice and stored at -70°C until assay (generally less than one week). The tissue was thawed on ice, and homogenized (1:230), with a polytron (Brinkmann Instruments, Westbury, NY) in WASH BUFFER (50 mM HEPES, 2.5 mM $MgCl_2$ and 2.0 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate (EGTA), pH adjusted to 7.4 at room temperature, with a saturated TRIS solution). The homogenate was washed twice via centrifugation (39,000 x g for 10 min., at 4°C.) The supernatant was discarded and the pellet resuspended the first time in wash buffer, and the second time in ASSAY BUFFER (WASH BUFFER containing 2 IU/ml adenosine deaminase (EC 3.5.4.4, calf spleen type X, Sigma Chemical Co., St. Louis, MO) and 0.1% ascorbate). The homogenate was then incubated at 37°C for 10 min., to remove endogenously bound 5-HT (Nelson et al., 1978), or adenosine (Bruns, 1980), and centrifuged as above. The final pellet was suspended in the initial volume of assay buffer and used immediately. The membrane homogenate for the adenylyl cyclase assay was prepared as described by De Vivo and Maayani (De Vivo and Maayani, 1986).

High Affinity Binding Assay. Radioligand binding experiments ([^{3}H]-R-PIA, (±)-[^{3}H]-8-OH-DPAT, [^{3}H]-BMY-7378), were carried out in duplicate in an assay volume of 1 ml consisting of 750 µl membrane homogenate (approximately 0.1 mg protein/ml), 50 µl

ASSAY BUFFER or masking ligand, 100 µl [^{3}H]-agonist, and 100 µl guanine nucleotide or ASSAY BUFFER. Competition of guanine nucleotide (Gpp(NH)p or GTPγS) for agonist binding was conducted at 6 different concentrations of [^{3}H]-agonist, 0.125 - 5 nM (24-93% initial receptor occupancy), thus in addition, constructing complete saturation binding isotherms. Nonspecific binding was defined by 1 µM 8-cyclopentyltheophylline (CPT; [^{3}H]-R-PIA), 1 µM (±)-8-OH-DPAT ([^{3}H]-BMY-7378), and 1 µM BMY-7378 ((±)-[^{3}H]-8-OH-DPAT), and was insensitive to the presence of guanine nucleotides. Incubations were initiated by the addition of the membrane preparation and were conducted for 60 min. at room temperature. Under these conditions, binding reached equilibrium within 1 hour and remained stable for at least 2 hours. Bound radioactivity was determined after rapid filtration through Whatman GF/C filter strips, using a Brandel cell harvester (Gaithersburg, MD). Unbound radioligand was removed with 3 x 5 ml of ice-cold WASH BUFFER. Specific binding was at least 90% of total binding. Filters were counted by liquid scintillation spectroscopy in Formula 963 (New England Nuclear, Boston, MA) at approximately 51% efficiency. Protein was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin as the standard.

Adenylyl Cyclase Assay. Adenylyl cyclase was assayed as described previously (Zgombick et al. 1989).

Materials. [^{3}H]-R-PIA (36 - 42 Ci/mmol), (±)-[^{3}H]-8-OH-DPAT (120 - 142 Ci/mmol), and [^{3}H]-BMY-7378 (36 Ci/mmol) were obtained from New England Nuclear (Boston, MA). CPT and (±)-8-OH-DPAT were obtained from Research Biochemicals Inc. (Natick, MA). Unlabeled BMY-7378 was synthesized by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT). All other reagents were obtained from Sigma Chemical Co., and were of the highest grade available.

Data Analysis. Saturation data were analyzed using the nonlinear regression analysis program LIGAND (Munson and Rodbard, 1980) Binding curves were best fit to a one site model, yielding K_d, B_{max} and n_H.

Guanine nucleotide inhibition of agonist binding and inhibition of forskolin-stimulated adenylyl cyclase were analyzed by fitting a four parameter logistic equation to the data. The equation used was:

$$R = [R_i-(R_i-R_o)]/[([A]/EC_{50})^n+1]$$

where,

R = The amount of [^{3}H]-agonist bound (pmol/mg protein) in the presence of a specified concentration of guanine nucleotide or the rate of adenylyl cyclase activity (pmol/min/mg protein) in the presence of a specific concentration of agonist.

R_o = The amount of [^{3}H]-agonist bound (pmol/mg protein) in the absence of guanine nucleotide or the rate of adenylyl cyclase activity in the absence of agonist (i.e., the basal level)

R_i = The amount of [^{3}H]-agonist bound (pmol/mg protein) in the presence of a saturating concentration of guanine nucleotide or the rate of adenylyl cyclase activity in the presence of a maximal concentration of agonist (E_{max}).

A = The concentration of guanine nucleotide or agonist mediating the response.

EC_{50} = The concentration of guanine nucleotide or agonist eliciting a half maximal inhibition of the response.

n = The slope index for the concentration response curve.

The density of sites sensitive to a saturating concentration of Gpp(NH)p (Fig. 6) can be described by the law of mass action and therefore was analyzed as a simple saturation, using the following equation:

$$B = B_{max} / 1+ (K_d/D)^n$$

Concentration response curves using the above equations were analyzed using the curve fitting program Kaleidagraph (Synergy Software, Reading, PA).

Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. K_d and EC_{50} (Fig. 3 A-C and 7A) values were fit by linear regression followed by a two tailed correlation analysis. The accepted level of significance was $p \leq 0.05$.

RESULTS

Relationship between drug relative efficacy and parameters of GTP concentration-inhibition curves of forskolin-stimulated adenylyl cyclase activity. Previous studies examining 5-HT_{1A} (De Vivo and Maayani, 1986) and AD A_1 (Ebersolt et al., 1983; Zgombick, 1989) receptor subtypes in rat hippocampal membranes, demonstrated the obligatory role of GTP in coupling these receptors to inhibition of forskolin-stimulated adenylyl cyclase activity. Illustrated in Fig. 1 are concentration-effect curves to GTP (10 nM-100 μM) each assayed in the presence of a saturating concentration (10 μM) of either 5-HT agonists (5-HT, (±)-8-OH-DPAT, buspirone or BMY 7378) or AD A_1 agonists (R-PIA or 2-phenylaminoadenosine (PAA)). The effect elicited in this preparation by 5-HT agonists is mediated through the 5-HT_{1A} receptor subtype, as the K_d value of spiperone (25 nM; De Vivo and Maayani, 1986) was independent of agonist tested (5-HT, (±)-8-OH-DPAT and buspirone) while the effect of 5-carboxamidotryptamine (5-CT), another 5-HT_{1A} agonist in this preparation, (unpublished data) was inhibited in a competitive manner by the partial 5-HT_{1A} agonists buspirone (unpublished data) or BMY 7378 (Yocca et al., 1987). Similarly, the effect elicited by either R-PIA or PAA is mediated through the AD A_1 receptor subtype because the same K_d value for CPT (10 nM; Zgombick et al., 1989; Craddock-Royal, 1991) was observed with each agonist.

Two parameters of the GTP concentration-effect curves, EC_{50} and slope index, were neither drug-efficacy nor receptor type dependent. The EC_{50} values determined in the presence of 5-HT_{1A}

agonists (0.7-2.7 μM) or AD A_1 agonists (0.6-2.3 μM) were independent of drug efficacy (p=0.27 and 0.62, respectively) and receptor type (p=0.44). Similarly, the slope indices of all curves (0.8-1.4) were not different from unity (p=0.93), and therefore were not receptor or drug efficacy dependent (p=0.86, AD A_1 and p=0.65, 5-HT_{1A}). In contrast, the third parameter, E_{max}, was both drug and receptor dependent. Because all drugs were tested at approximately 100 times the EC_{50} (De Vivo and Maayani, 1986, Zgombick et al., 1989), the inhibition observed at 10 μM GTP should compare to the reported E_{max} values calculated from drug concentration-effect curves in the presence of 10 μM GTP. Confirming our previous reports (De Vivo and Maayani, 1986; Yocca et al., 1987), the E_{max} values of the two azapirones analogs, buspirone (17% inhibition) and BMY 7378, (9%) were lower ($p \leq 0.01$) than the E_{max} values exhibited by 5-HT (31%) or by (±)-8-OH-DPAT (22%) . Similarly, PAA exhibited a lower E_{max} value (11%; $p \leq 0.001$) than that of R-PIA (41%) (Zgombick et al., 1989; Fig.1). Interestingly, among all drugs tested, the maximal agonist-mediated inhibition of forskolin-stimulated adenylyl cyclase activity was achieved by R-PIA (40%), which is similar to the maximal inhibition achieved by direct activation of the G-protein with 10 μM Gpp(NH)p (data not shown).

Relationship between inhibition of forskolin-stimulated adenylyl cyclase and high affinity binding of AD A_1 and 5-HT_{1A} agonists. A comparison between characteristics of concentration-effect curves and those of concentration-binding curves to R-PIA, (±)-8-OH-DPAT and BMY-7378 is shown in Fig. 2.

Consistent with results presented in Fig. 1, the rank order of E_{max} (%) values was R-PIA (38) > (±)-8-OH-DPAT (23) > BMY-7378 (8) The EC_{50} (nM) exhibited a reverse order of potency, 55, 17, and 6 respectively. Binding parameters determined in three independent experiments are presented in Table 1. The tested drugs exhibited similar K_d values (nM), 0.35 ± 0.02 (R-PIA), 0.36 ±0.04 ((±)8-OH-DPAT), and 0.40 ± 0.07 (BMY-7378), but different B_{max} values (pmol/mg protein): 1.44 ± 0.16 (R-PIA), 1.04 ± 0.03 ((±)-8-OH-DPAT), 0.65 ± 0.08 (BMY-7378) (n=3, with each individual repetition in a common membrane preparation). Different EC_{50}/K_d ratios were exhibited by the three drugs: 160 (R-PIA), 72 ((±)-8-OH-DPAT) and 10 (BMY-7378). Relative drug efficacy has been proposed to be proportional to this ratio (Kent et al, 1980). The Hill coefficients were not significantly different from unity for the three drugs.

Gpp(NH)p decreased the B_{max} values of [^{3}H]-agonist binding. Two 5-HT_{1A} agonists, (±)-8-OH-DPAT and BMY-7378, and the AD A_1 agonist R-PIA were available as tritiated ligands, which enabled a comparative study of their receptor binding sites as well as the effect of guanine nucleotides on the binding of the [^{3}H]-agonists to their respective receptor sites. Illustrated in Fig. 3 are concentration-binding curves (assayed using the same membrane preparation in the absence and presence of a saturating concentration of Gpp(NH)p (100 μM)) and Rosenthal (Scatchard) transformations for each [^{3}H]-agonist. Confirming previous reports, rat hippocampal membranes displayed a high density of both 5-HT_{1A} (Palacios et al., 1987) and AD A_1 (Erfurth and Reddington, 1986)

receptor binding sites. Intriguingly, the B_{max} values of (±)-8-OH-DPAT-sensitive [^{3}H]-BMY 7378 sites were consistently lower than those observed with BMY 7378-sensitive (±)-[^{3}H]-8-OH-DPAT sites. The three populations of receptor binding sites were only partially sensitive to 100 μM Gpp(NH)p (Fig. 3) as reflected by the partial decrease of the B_{max} values (pmol/mg protein): (±)-[^{3}H]-8-OH-DPAT: 1.04 to 0.61 (41%); [^{3}H]-BMY 7378: 0.60 to 0.41 (32%); and [^{3}H]-R-PIA): 1.26 to 0.67 (47%). The other two binding parameters of the concentration-binding curves, K_d and slope index, appeared to be unaffected by Gpp(NH)p (Fig. 3).

A complete characterization of the effect of Gpp(NH)p on [^{3}H]-agonist binding was done by determining the K_d, B_{max} and slope index of the bound [^{3}H]-agonist in the presence of fifteen different Gpp(NH)p concentrations (1 nM-100 μM). At each Gpp(NH)p concentration a six point concentration-binding curve (0.09-5.0 nM) was constructed. This experimental design enabled us to characterize the mutual effects of [^{3}H]-agonist and Gpp(NH)p binding at a wide range of their concentrations. As could be predicted from the data shown in Fig. 3, the K_d and the slope index values of sites labeled in the presence of Gpp(NH)p were unaffected by increasing Gpp(NH)p concentrations, while the B_{max} values of these sites were substantially affected by Gpp(NH)p: a gradual increase of the Gpp(NH)p concentrations decrease monotonically the B_{max} of all tritiated drugs in a saturable manner (Fig. 4). The maximal value of this effect occurred above 10 μM Gpp(NH)p with each of the [^{3}H]-agonists. When fit to the logistic equation (Materials and Methods) the largest decrease in B_{max} values was observed with [^{3}H]-R-PIA

(52%) and the lowest with [^{3}H]-BMY 7378 (37%) (Fig. 4, A-C). The midpoint value (IC_{50}, μM) was the highest for [^{3}H]-R-PIA (1.30) and lower for (±)-[^{3}H]-8-OH-DPAT (0.35) and [^{3}H]-BMY 7378 (0.47).

Attenuation of [^{3}H]-agonist binding by Gpp(NH)p: relationship between the parameters of Gpp(NH)p concentration-effect curves and receptor occupancy and agonist relative efficacy. The gradual decrease of B_{max} values with the increase of Gpp(NH)p concentrations (Fig.4) confirms previous reports on the allosteric attenuation by guanine nucleotides of (±)-[^{3}H]-8-OH-DPAT binding (Hall et al., 1985; Schlegel and Peroutka, 1986) and [^{3}H]-R-PIA (Yeung and Green, 1983; Ukena et al., 1984). However, the relationship between receptor occupancy and characteristics of the Gpp(NH)p effect on [^{3}H]-agonist binding have not been reported. To investigate the possibility that Gpp(NH)p attenuation of [^{3}H]-agonist binding is modulated by receptor occupancy, parameters of the Gpp(NH)p attenuation curves were assayed at a low receptor occupancy level (24%; Fig. 5) and compared to parameters derived from data shown in Fig. 4. As shown in Fig. 5, the maximal values of the Gpp(NH)p attenuation were similar across the two drugs at the low (Fig. 5) and the high (Fig. 4) occupancy levels: both plateaued at approximately 50%. Similarly, the slope index values of the Gpp(NH)p attenuation curves for a given drug appeared to be similar between the two levels of receptor occupancy. Fig. 5 illustrates that attenuation of (±)-[^{3}H]-8-OH-DPAT binding was observed at 10 nM Gpp(NH)p, and four log units of Gpp(NH)p concentrations were required to reach a plateau level around 10 μM

Gpp(NH)p. In contrast, attenuation of [^{3}H]-R-PIA binding did not occur until approximately 100-200 nM Gpp(NH)p, which was the IC_{50} of Gpp(NH)p for (±)-[^{3}H]-8-OH-DPAT attenuation, and plateaued well within two log units (100 nM-10 μM). Consequently, the slope index of the two curves differed ($p \leq 0.05$): 0.67 ((±)-[^{3}H]-8-OH-DPAT) vs 1.35 ([^{3}H]-R-PIA). Similar behavior was observed for the Gpp(NH)p effect on the B_{max} values of these drugs (Fig. 4), where slope indices were 0.74 and 1.09, respectively.

Unexpectedly, the IC_{50} values of the Gpp(NH)p attenuation curves were significantly higher at the maximal receptor occupancy (Fig. 4), 0.35 μM for (±)-[^{3}H]-8-OH-DPAT and 1.3 μM for [^{3}H]-R-PIA, than those observed at 24% of initial receptor occupancy, 0.13 μM and 0.57 μM, respectively (Fig. 5). This observation suggests that the Gpp(NH)p effect may be modulated by the degree of initial receptor occupancy by the [^{3}H]-agonist. To test this possibility, data collected from saturation experiments shown in Fig. 3 were expressed as concentration effect curves to Gpp(NH)p. The results of this transformation are illustrated in Fig. 6 (A-C) for three of the six different levels of receptor occupancy, and are summarized in Table 2. Table 2 shows that for a given drug, the slope index was not affected by varying its concentration. However, similar to results observed with the highest (Fig. 4) and the lowest (Fig. 5) [^{3}H]-agonist concentration, a consistent difference in the slope index was observed with all [^{3}H]-R-PIA concentrations (uniyt to greater than unity; 1 - 1.3) when compared to each concentration of either (±)-[^{3}H]-8-OH-DPAT or [^{3}H]-BMY 7378 (approximately 0.7) (Fig 6 A-C; Table 2).

The effect of agonist concentration on the maximal attenuation of [^{3}H]-agonist binding (E_{max}) by Gpp(NH)p was analyzed in two ways. When expressed as percent of [^{3}H]-agonist bound in the absence of Gpp(NH)p, the E_{max} was independent of [^{3}H]-R-PIA concentration (about 50%), but decreased progressively ($p \leq 0.05$) with increasing (±)-[^{3}H]-8-OH-DPAT (59% to 51%) or [^{3}H]-BMY 7378 (51% to 31%) concentration (Fig. 6 A-C; Table 2). However, when E_{max} values were expressed as density of sites (pmol/mg protein) that are sensitive to Gpp(NH)p, they increased with increasing [^{3}H]-agonist concentration in a saturable manner which could be used to construct concentration-dependence curves of Gpp(NH)p-sensitive sites (Fig. 7). Fig. 7 shows that at all concentrations of [^{3}H]-agonist examined the amount of sites sensitive to Gpp(NH)p was greatest for [^{3}H]-R-PIA and lowest for [^{3}H]-BMY 7378. Moreover, the K_d values of these populations of sites were indistinguishable ($p=0.13$, 0.48, and 0.76, [^{3}H]-R-PIA, (±)-[^{3}H]-8-OH-DPAT, and [^{3}H]-BMY 7378 respectively) from those of the entire population of sites (e.g., Fig. 3).

The IC_{50} of the Gpp(NH)p concentration curves increased progressively with increasing [^{3}H]-agonist concentration, which can be seen from a dextral "shift" of the concentration-effect curve (Fig. 6 and Table 2). Assuming a linear relationship, a statistically significant correlation was observed between IC_{50} and [^{3}H]-agonist concentration for the three [^{3}H]-agonists (Fig. 8A). As shown in this figure, the slope values of the linear regressions for (±)-[^{3}H]-8-OH-DPAT and [^{3}H]-BMY 7378 were congruent, while that of the AD A_1 agonist was significantly higher (Fig. 8A). If the IC_{50} values are examined not as a function of [^{3}H]-agonist concentration, but were

related to initial receptor occupancy, the increase was not apparent until 50-60% occupancy, but increases steeply at higher levels of initial receptor occupancy (Fig. 8B).

The GTPγS effect is qualitatively similar to that of Gpp(NH)p. The allosteric interaction between GTPγS and [^{3}H]-agonist binding sites were qualitatively similar to those observed between Gpp(NH)p and [^{3}H]-agonist radioligand (Tables 2 and 3). The IC_{50} values of GTPγS concentration-effect curves progressively increased with increasing receptor occupancy, however, for equal concentrations of [^{3}H]-agonist, the IC_{50} values obtained with GTPγS were 10-30 times lower (Fig. 9). A substantial but apparently constant (approximately 50%) subpopulation of all [^{3}H]-agonist binding sites exhibited insensitivity to both guanine nucleotides. The portion of [^{3}H]-agonist binding sites which were sensitive to GTPγS increased in a saturable manner, with increasing [^{3}H]-agonist concentration exhibiting a similar rank order of maximal density with both guanine nucleotides: [^{3}H]-R-PIA > (±)-[^{3}H]-8-OH-DPAT > [^{3}H]-BMY-7378.

DISCUSSION

Relationship of High Affinity Binding of 5-HT_{1A} and AD A_1 Agonists to Their Inhibition of Forskolin Stimulated Adenylyl Cyclase. Concentration-binding curves of the three tested drugs are located to the left of the corresponding concentration-response curves (Fig. 2). A proposal formulated to explain these differential locations is the existence of at least two distinct, hormone-related, receptor affinity states: a high affinity ternary complex (hormone/receptor/G-protein), and a low affinity state, formed from the ternary complex by binding of guanine nucleotides to the G-protein. The ternary complex model was originally proposed for the G_s-linked beta-adrenergic receptor (De Lean et al., 1980) and has since been proposed for other receptors coupled to effector systems through G-proteins, i.e., dopamine (Zahniser and Molinoff, 1978; Hamblin et al., 1984), 5-HT_1 (Schlegel and Peroutka, 1986; Stratford et al., 1988, 5-HT_2 (Strange, 1988; Strange, 1990), muscarinic (Ehlert, 1985; Galper et al., 1987), and opioid (Itzhak, 1989; Werling et al., 1988).

The ternary complex model as proposed, allows for at least a portion of receptors to be spontaneously 'precoupled' to the G-protein, without hormone (De Lean et al., 1980). Agonists bind to and stabilize this complex. This possibility may be the case for the two receptors chosen in this study, because solubilization of AD A_1 (Stiles, 1988) and 5-HT_{1A} (El Mestikawy et al., 1988; Emerit et al., 1990) receptors has shown them to be associated with G-proteins.

The G_i-linked AD A_1 and 5-HT_{1A} receptors (Zgombick et al, 1989) appear to form a stable high affinity ternary complex with radiolabeled hormones H-R-G without guanine nucleotides (Fig. 3). Binding of guanine nucleotides to the G-protein decreases the affinity of the hormone for the ternary complex (De Lean et al., 1980). The H-R-G ternary complex is rapidly destabilized by guanine nucleotides, such as Gpp(NH)p and GTPγS, with a half-life of less than 10 seconds (data not shown). The high affinity state of a receptor has been proposed to be an essential intermediate in activation of adenylyl cyclase (Stadel et al., 1980). Destabilization of the high affinity state, induced by guanine nucleotides, is associated with an increase of functional activity (Lefkowitz et al., 1976b).

Many attempts have been made to equate a particular receptor affinity state with its functionality in a cell. Several reports have shown an apparent congruency between EC_{50} values in a variety of receptor systems and the dissociation constant values for low affinity binding (K_L) in both cell free membrane preparations (Ehlert, 1985; Jakobs et al, 1978) and intact cells (Nathanson, 1983; Ströher et al, 1989). In COS-7 cells expressing the human gene of the 5-HT_{1A} receptor (G-21), binding of (±)-[^{3}H]-8-OH-DPAT yielded two affinity states (K_H and K_L; Fargin et al., 1988). The K_L (nM; 22, 63, and 97, (±)-8-OH-DPAT, 5-HT, and buspirone, respectively) was similar to the EC_{50} of the agonist in the inhibition of forskolin-stimulated adenylyl cyclase (24, 109, 170; De Vivo and Maayani, 1986). Similarly, the shallow binding curves of three AD A_1 agonists to the antagonist [^{3}H]-DPCPX sites, were deconvoluted by a two state model; where K_L

values approximated the EC_{50} for the inhibition of adenylyl cyclase activity (Ströher et al., 1989). Using the same compounds in intact cardiac myocytes has shown a single low affinity state with K_i values corresponding to EC_{50} values for the inhibition of isoproterentol-stimulated cyclic AMP accumulation (Martens et al., 1988). In this study, we have attempted to correlate agonist-related high and low affinity states of the 5-HT_{1A} receptor subtype. In addition we attempted to identify similarities and differences in these characteristics between the 5-HT_{1A} and the AD A_1 receptor subtypes. The high affinity state was studied by binding of [^{3}H]-agonists, and the low affinity state was examined in two ways: i). loss of H-R-G, in the presence of guanine nucleotides, and ii). a functional assay (inhibition of forskolin-stimulated adenylyl cyclase activity), where the affinity of the agonists for the receptors was 10-170 times lower than in radioligand binding (Fig. 2).

In receptor systems where a receptor reserve does not exist, the EC_{50} of a given drug is equal to its dissociation constant, K_a (Kenakin, 1987). If $EC_{50} = K_L$, and if $EC_{50} = K_a$, then $K_a = K_L$. Partial alkylation of the 5-HT_{1A} receptor with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), *in vitro*, which did not change the EC_{50} for (±)-8-OH-DPAT and other 5-HT_{1A} agonists, decreased the maximal inhibition of forskolin-stimulated adenylyl cyclase activity (Yocca et al, 1990). In contrast, the lack of an effective alkylating agent of the AD A_1 receptor has made similar analysis impossible. Yet, Ströher et al. (Ströher et al, 1989), have proposed, based upon comparable EC_{50} values for agonist inhibition of cerebral cortical adenylyl cyclase

activity and K_i values for the low affinity state of the AD A_1 receptor, a lack of receptor reserve in guinea pig cortical membranes.

Effects of Guanine Nucleotides on Receptor / G-protein interaction. The 5-HT_{1A} and AD A_1 receptors exhibit a high density in rat hippocampal membranes, where they mediate a concentration-dependent inhibition of adenylyl cyclase activity (Figs. 1 and 2). Because these receptor subtypes are found in the same region, and since they activate at least two common effectors in a non-additive manner (inhibition of forskolin-stimulated adenylyl cyclase activity and activation of K^+ channels), we have postulated that they may share components of the signal transduction pathway (Zgombick et al., 1989). An advantage of a cell free system is that one can determine agonist-related characteristics of both affinity states of the receptor and try to relate them to molecular properties of the drugs, such as agonist relative efficacy and differences in these properties which may exist as a function of receptor type.

To determine the effect of G-protein occupation by guanine nucleotide on parameters of agonist binding (K_d, B_{max}, and Hill coefficient), we have constructed binding curves for AD A_1 ([^{3}H]-R-PIA) and 5-HT_{1A} ((±)-[^{3}H]-8-OH-DPAT and [^{3}H]-BMY-7378) agonists in the presence and absence of fifteen different concentrations of Gpp(NH)p, in the same hippocampal membrane preparations. In the absence of Gpp(NH)p, the B_{max} value (pmol/mg protein) of [^{3}H]-R-PIA (1.44) was consistently greater than that of (±)-[^{3}H]-8-OH-DPAT (1.04) and [^{3}H]-BMY-7378 (0.65), showing a possible greater density

of AD A_1 receptors in rat hippocampal membranes. Also, since the E_{max} value of BMY-7378 is less than that of (±)-8-OH-DPAT, it may suggest that the agonist relative efficacy may be related to the density of the high affinity ternary complex formed. This relationship was previously proposed for the G_s-linked beta adrenergic receptor, where the density of ternary complex formed was related to agonist intrinsic activity (Kent et al., 1980). If this is true also for the 5-HT_{1A} and the AD A_1 receptors, then the greater density of H-R-G, makes available a larger amount of complex for destabilization and for production of a functional response (Figs. 1, 2).

Guanine nucleotides have been reported to affect agonist binding to AD A_1 and 5-HT_{1A} receptors by either changing affinity (Schlegel and Peroutka, 1986; Stiles, 1988), density of binding sites (Herrick-Davis and Titeler, 1988), or both affinity and density (Yeung and Green, 1983; Harrington and Peroutka, 1990). The experimental conditions of our binding assay i.e., agonist concentrations restricted to measure only the high affinity component, presence of Mg^{++} (2.5 mM), and lack of guanine nucleotides (membranes washed multiple times in 2.5 mM Mg^{++}), assures measurement of mainly H-R-G. In contrast, the presence of GTP in the adenylyl cyclase assay promotes formation (even in the presence of Mg^{++}) of the low affinity state of the receptor (Figs. 1, 2).

The addition of Gpp(NH)p did not change the affinity of any of the three [^{3}H]-agonists for their respective receptor sites, but it

decreased the observed B_{max} values (Fig 3 and 4). This is in agreement with the theory of destabilization of H-R-G by Gpp(NH)p. Other reports which have used higher concentrations of (±)-[^{3}H]-8-OH-DPAT, which probably occupied a significant portion of the low affinity state of 5-HT_{1A} receptors (Schlegel and Peroutka, 1986; Harrington and Peroutka, 1990), have shown both a guanine nucleotide-dependent decrease in B_{max} and increase in K_d values. To detect a change in agonist affinity in our assay, K_H/K_L would have to be well within the concentration of [^{3}H]-agonist tested (0.125 nm to 5.0 nM). For [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT, this is very unlikely, as the ratio of EC_{50}/K_d is 160 and 72, respectively, and the highest concentration of [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT would not occupy a significant portion of the low affinity state of the receptor. However, for [^{3}H]-BMY-7378, the EC_{50}/K_d ratio is significantly lower, approximately 10. For example, at 0.5 nM [^{3}H]-BMY-7378, the occoupancy of the low affinity state is about 35% of its B_{max}. This may explain the trend to an increase in the apparent observed K_d of [^{3}H]-BMY-7378 with the increase of Gpp(NH)p concentration (Fig. 4).

While the maximal attenuation of [^{3}H]-R-PIA binding by Gpp(NH)p was about 50% at all concentrations of [^{3}H]-R-PIA, the maximal inhibition of [^{3}H]-BMY-7378 binding and to a lesser extent, (±)-[^{3}H]-8-OH-DPAT, decreased as their concentrations increased. This presumably occurs because for both BMY-7378 and (±)-8-OH-PAT, the high and a portion of the low affinity state can be labeled within the concentration range tested, which is not the case for R-

PIA (Fig. 2, Table 1). At the higher level of receptor occupancy, the agonist should bind to a finite amount of high, but to an increasing proportion of low affinity sites, and a lesser proportion of the bound agonist can be converted to the low affinity state. A similar finding has been shown for the opioid agonist [^{3}H]-DADLE in NG 108-15 membranes (Ott and Costa, 1989).

It could be expected that Gpp(NH)p would completely destabilize all H-R-G formed because of at least two reasons: 1) The ratio of G_i / receptor in brain tissue has been shown to be larger than unity (Neubig et al., 1988) and 2). Since all receptor sites appear to be homogeneous for agonist binding (Fig. 2, 3), the entire H-R-G population should be equally affected by Gpp(NH)p. However, the addition of guanine nucleotide converted only a portion of these sites into the low affinity state (40-60%, Fig. 3, 4, 6, Table 2). This phenomenon has also been reported by others (Herrick-Davis and Titeler, 1988; Rydelek et al., 1990; Harrington and Peroutka, 1990). Therefore, H-R-G, can be divided into two populations: a population that is sensitive to guanine nucleotides (Fig. 7) and a population that is insensitive (Fig. 3) to guanine nucleotides. For any of the three [^{3}H]-agonists, both populations exhibit similar K_d values (Fig. 3, Fig. 7). The lack of complete sensitivity to Gpp(NH)p may reflect the introduction of artifact from the preparation of membranes. Stroher et al., has shown that solubilization of the AD A_1 receptor enables complete conversion of the high affinity state into the low affinity state (Ströher et al., 1989). Similarly, binding of [^{3}H]-R-PIA and (±)-^{3}H]-8-OH-DPAT to 15 μ sections of rat brain or human post-mortem

brain has shown near complete loss of binding in the presence of guanine nucleotides (CHAPTER 3). Similar results were reported in rat brain for the 5-HT_{1A} receptor, using [^{3}H]-eltoprazine (Sijbesma et al, 1990), the AD A_1 receptor, using [^{3}H]-cyclohexyladenosine (Fastbom and Fredholm, 1990) and the AD A_2 receptor using [^{3}H]-CGS-21680 (Parkinson and Fredholm, 1990).

Studies in intact cells have suggested the presence of a single class of low affinity agonist binding sites. This has been shown for the AD A_1 receptor system in cardiac myocytes (Martens et al., 1988) and intact rat adipocytes (Lohse et al, 1986). Characterization of AD A_1 receptors in DDT_1 MF_2 smooth muscle cell membranes or intact cells has shown the high affinity state to exist in only the membrane preparation, while the low affinity state exists and is quantitatively similar in both the membrane preparation and intact cells (Gerwins et al., 1990a). The inability to detect the high affinity state in intact cells does not necessarily suggest it is an experimental artifact, in fact, Lefkowitz and colleagues (Stadel et al., 1980) have proposed that the high affinity state is a prerequisite for functional activity. The absence of detectable high affinity binding in intact cells may suggest the abundance of endogenous guanine nucleotides and the rapidity in their binding and conversion into the low affinity functional state.

Under our assay conditions, Gpp(NH)p sensitive binding increased with [^{3}H]-agonist concentration. If the density of H-R-G formed is related to agonist efficacy (Kent et al, 1980 and Fig. 2),

then the density of sites sensitive to Gpp(NH)p may reflect agonist efficacy (Fig. 7). A similar phenomenon has been reported for two other 5-HT_{1A} partial agonists, where GTPγS decreased the B_{max} of [^{3}H]-NAN-190 and [^{3}H]-spiroxatrine, both of which are weak partial agonists, significantly less than that of the full agonist (±)-[^{3}H]-8-OH-DPAT (Rydelek et al., 1990). As agonists of very low intrinsic activity may appear both functionally and behaviorally as antagonists, [^{3}H]-agonist sensitivity to guanine nucleotides may be equated with agonist efficacy in an effectorless system.

Allosteric Interactions Between Hormone Receptors and Guanine Nucleotides. We assume that hormone (agonist) receptor binding sites and guanine nucleotide binding sites are distinct sites on different proteins. Therefore, the observed mutual effects of hormone and guanine nucleotide binding reported here are proposed to be a reflection of allosteric interactions. We characterized this allosterism by measuring their mutual effects at different ligand concentrations (Fig. 4 and 6). Binding of all agonists was attenuated by Gpp(NH)p, in a concentration-dependent and saturable manner (Fig. 6). At 24% initial receptor occupancy, the IC_{50} was 0.1 μM for (±)-[^{3}H]-8-OH-DPAT, while the attenuation of [^{3}H]-R-PIA binding was not measureably detected until 0.1 μM Gpp(NH)p. This higher sensitivity and shallowness of the curve of bound [^{3}H]-5-HT_{1A} agonists to guanine nucleotides compared to that of bound [^{3}H]-R-PIA (Table 2, Fig. 5, 6) may indicate intriguing differences occurring between the ternary complexes formed by the two receptors. For example, while both receptors may share a common pool of G-

proteins (Zgombick et al., 1989), the 5-HT_{1A} system may have access to other types of pertussis toxin-sensitive G-proteins. The observed differences in the slope index may also suggest receptor-related differences in the stoichiometry of receptor/G-protein interactions. Guanine nucleotide inhibition of opioid agonist binding also displays shallow slopes (Ott and Costa, 1989) compatible with multiple affinity states of the G-protein. It has been hypothesized that for the opioid system (Ott and Coata, 1989) the differing affinities occur as the result of a high affinity component caused by the nucleotide binding to the G-protein holocomplex $\alpha\beta\gamma$, while the low affinity component is the result of nucleotide binding to the free α subunit. As the two different G_i-protein linked receptors in this study which in all likelihood are capable of utilizing at least a common portion of G-proteins, exhibit such diversity in Gpp(NH)p attenuation curves, this proposal alone is an unlikely explanation.

Binding parameters of the agonists and of Gpp(NH)p which were mutually affected are summarized in Table 4. The IC_{50} values of the Gpp(NH)p curves increased as receptor initial occupancy increased. This may result from a complex stoichiometry between the coupling of the receptor and G-protein. One possible way to test this proposal is to introduce defined proportions of receptors and G-proteins into the same clonal cell line and then test the allosteric interaction between hormone and guanine nucleotide binding. In summary, different G_i-linked receptors appear to display different characteristics of allosteric interactions between hormone and guanine nucleotide binding. This allosteric interaction is proposed as

a model to study biochemical characteristics associated with drug efficacy.

Fig 1. GTP concentration-effect curves for the inhibition of forskolin-stimulated adenylyl cyclase activity in the presence of AD A_1 agonists (A) and 5-HT_{1A} agonists (B), each at 10 μM. The basal level of forskolin-stimulated adenylyl cyclase was 174 ± 14 pmol/min/mg protein. Data shown in the presence of the indicated agonists are values above the basal GTP activity. Data represent percent of control forskolin (10 μM) stimulated adenylyl cyclase activity. Curves were fit to the logistic equation described in Materials and Methods, which generated the following parameters (data shown in parentheses represent mean ± SE for three separate experiments, each performed in triplicate):

Agonist	E_{max} (%)	EC_{50} (μM)	slope index
R-PIA	41 (42±1)	1.52 (1.58±0.09)	1.09 (1.21±0.10)
PAA	11 (13±1)[a]	2.30 (1.30±0.52)	0.85 (1.28±0.37)
5-HT	31 (30±2)	1.84 (1.98±0.20)	1.18 (1.16±0.09)
(±)-8-OH-DPAT	22 (22±2)	0.92 (1.14±0.18)	1.30 (1.37±0.03)
buspirone	17[b] (16±1)	0.70 (1.81±0.74)	0.97 (1.26±0.30)
BMY-7378	9[b,c] (9±1)	1.49 (1.16±0.21)	1.42 (1.06±0.20)

[a] Statistically different ($p \leq 0.001$) from the E_{max} obtained with the full agonist R-PIA.

[b] Statistically different ($p \leq 0.01$) from the E_{max} obtained with the full agonist 5-HT.

[c] Statistically different ($p \leq 0.01$) from the E_{max} obtained with the agonist (±)-8-OH-DPAT.

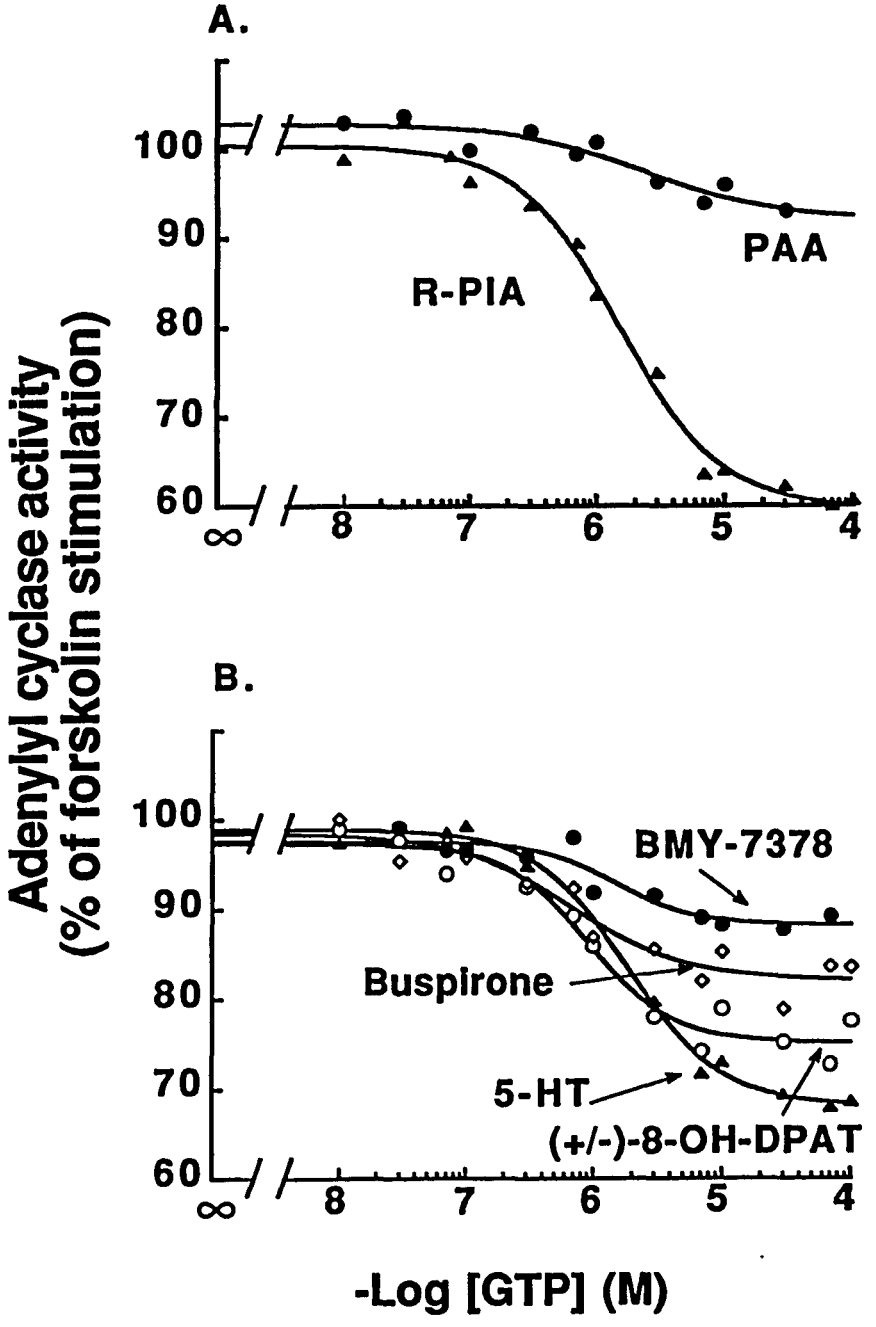

A.
100
90
80
70
60
∞
8
7
6
5
4
PAA
R-PIA
B.
BMY-7378
Buspirone
5-HT
(+/-)-8-OH-DPAT
Adenylyl cyclase activity
(% of forskolin stimulation)
-Log [GTP] (M)

TABLE 1

Parameters of high affinity specific binding and inhibition of forskolin-stimulated adenylyl cyclase by the adenosine A_1 agonist R-PIA and the 5-HT_{1A} agonists (±)-8-OH-DPAT and BMY-7378.

Experiments were performed and data analyzed as described in Materials and Methods. For radioligand binding, values shown represent mean ± standard error of three separate determinations. For each repetition, experiments were performed in duplicate, in the same membrane preparation. For the adenylyl cyclase assay, values shown represent four to six determinations performed in triplicate. Values listed in parentheses represent arithmetic means of K_d and EC_{50} values.

	AGONIST		
	R-PIA	(±)-8-OH-DPAT	BMY-7378
Binding			
B_{max} (pmol/mg protein)	1.44 ± 0.16[a]	1.04 ± 0.03	0.65 ± 0.08[a]
pK_d	9.45 ± 0.03 (0.35 nM)	9.45 ± 0.05 (0.36 nM)	9.41 ± 0.10 (0.40 nM)
n_H	1.04 ± 0.05	1.10 ± 0.05	0.96 ± 0.15
Adenylyl cyclase			
E_{max} (% inhibition)	38 ± 1.08[a]	23 ± 1.45	6 ± 2[a]
pEC_{50}	7.26 ± 0.06 (56 nM)	7.60 ± 0.17 (26 nM)	8.73 ± 0.76[b] (4 nM)
slope index	1.09 ± 0.08	1.05 ± 0.13	0.90 ± 0.17
EC_{50}/K_d	160	72	10

[a]Significantly different from values obtained with (±)-8-OH-DPAT ($p \leq 0.05$, ANOVA and Duncan's multiple range test)
[b]Data in agreement with K_b values reported by Yocca (Yocca et al., 1990) using BMY-7378 as an antagonist of 5-CT mediated inhibition of forskolin-stimulated adenylyl cyclase.

Fig. 2. Representative concentration-binding curves (■, ●, ▲) and agonist-mediated inhibition of forskolin-stimulated adenylyl cyclase activity (□, 0, Δ) by R-PIA (A), (±)-8-OH-DPAT (B), and BMY-7378 (C) in rat hippocampal membranes. Each data point represents the mean of duplicate (binding) or triplicate (adenylyl cyclase) determinations, with standard deviations (not shown) less than 5 % of the mean. Estimates of K_d and B_{max} values were obtained by non-linear regression analysis (Materials and Methods). For the representative experiment shown, calculated values were:

Drug	[^{3}H]-Agonist Binding			Adenylyl Cyclase Activity		
	K_d (nM)	B_{max} (pmol/mg protein)	n_H	E_{max} (%)	EC_{50} (nM)	slope index
R-PIA	0.34	1.26	1.04	40	55	0.90
(±)-8-OH-DPAT	0.33	1.04	1.14	24	17	1.19
BMY-7378	0.27	0.60	0.80	8	6	0.70

The above values yield a ratio of EC_{50}/K_d = 157, 52, 22. The basal level of forskolin-stimulated adenylyl cyclase was 259 ± 37 pmol/min/mg protein. Table 1 summarizes the values of all data collected.

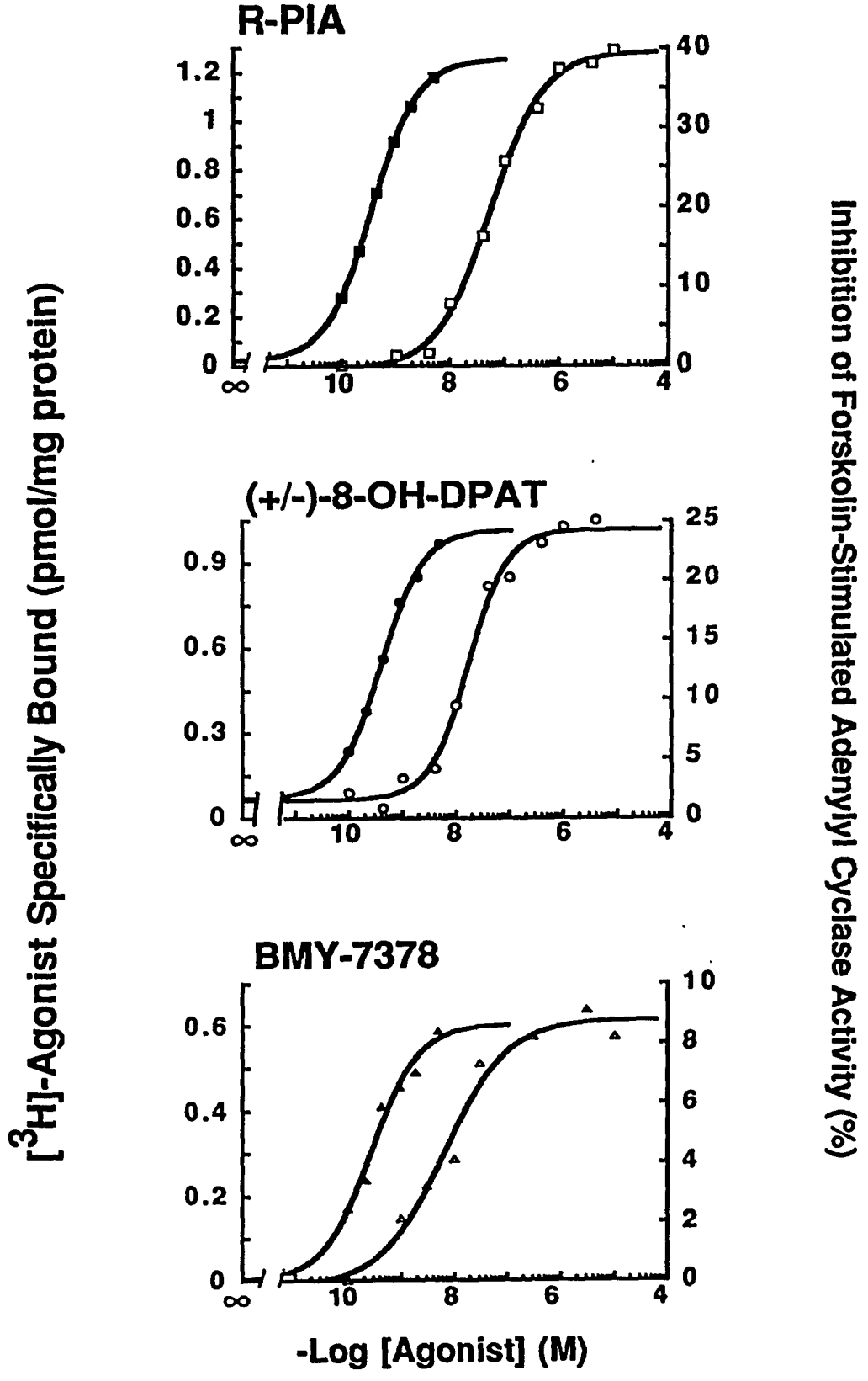
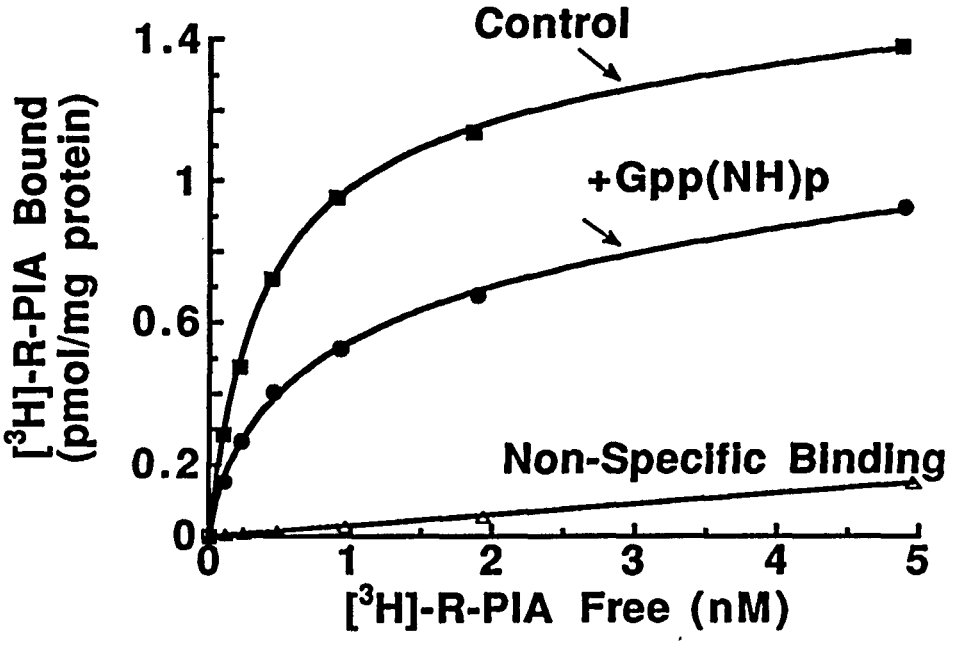
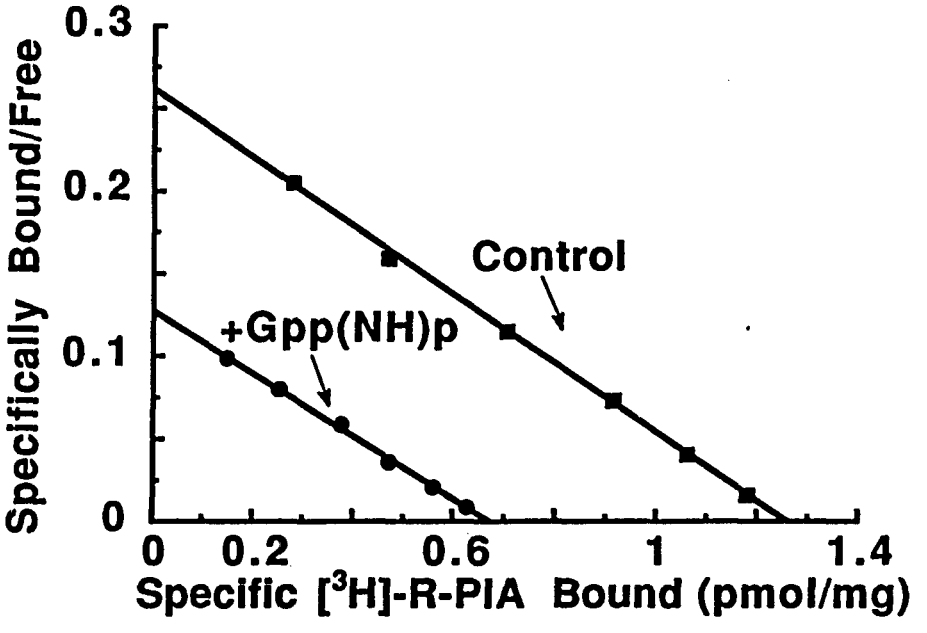

R-PIA
(+/-)-8-OH-DPAT
BMY-7378
[3H]-Agonist Specifically Bound (pmol/mg protein)
Inhibition of Forskolin-Stimulated Adenylyl Cyclase Activity (%)
-Log [Agonist] (M)

Fig. 3. Representative concentration-binding curves and Rosenthal transformation of [^{3}H]-R-PIA (A), (±)-[^{3}H]-8-OH-DPAT (B), and [^{3}H]-BMY-7378 (C). The assays were done on the same rat hippocampal membrane preparation, in the absence and presence of 100 μM Gpp(NH)p. Non-specific binding was defined in the presence of 10 μM CPT ([^{3}H]-R-PIA), 1 μM BMY-7378 ((±)-[^{3}H]-8-OH-DPAT), or 1 μM (±)-8-OH-DPAT ([^{3}H]-BMY-7378). Data points shown represent the mean of duplicate determinations, with standard deviations less than 5% of the mean. Estimates of K_d and B_{max} values were obtained from non-linear regression analysis as described in Materials and Methods and are summarized in the table below and were replicated an additional two times yielding similar results:

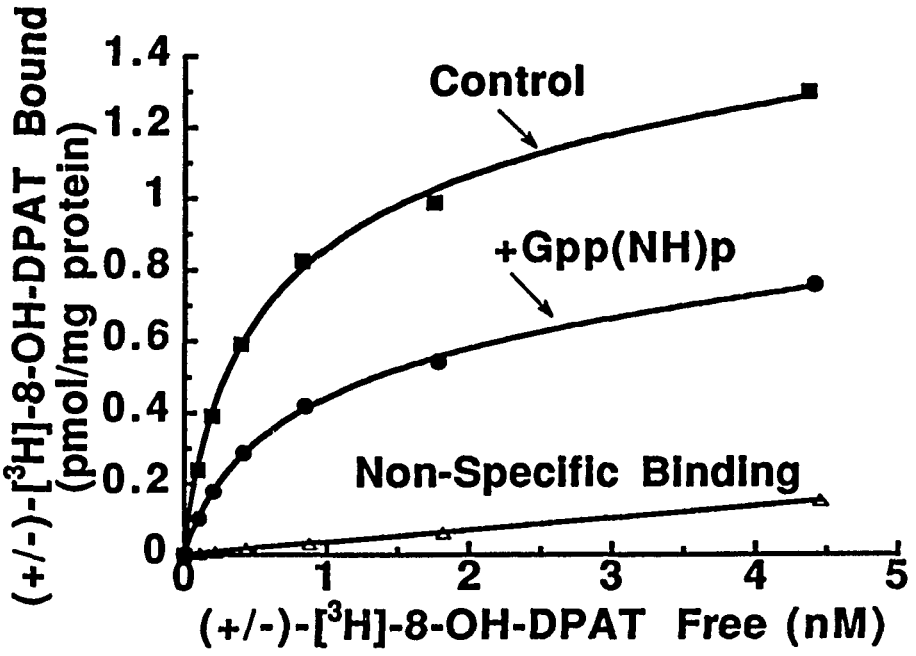
[^{3}H]-Agonist	K_d (nM)		B_{max} (pmol/mg)		n_H	
	Control	+Gpp(NH)p	Control	+Gpp(NH)p	Control	+Gpp(NH)p
R-PIA	0.34	0.38	1.26	0.67	1.04	1.15
(±)-8-OH-DPAT	0.33	0.55	1.04	0.61	1.14	1.04
BMY-7378	0.27	0.45	0.60	0.41	0.80	1.15

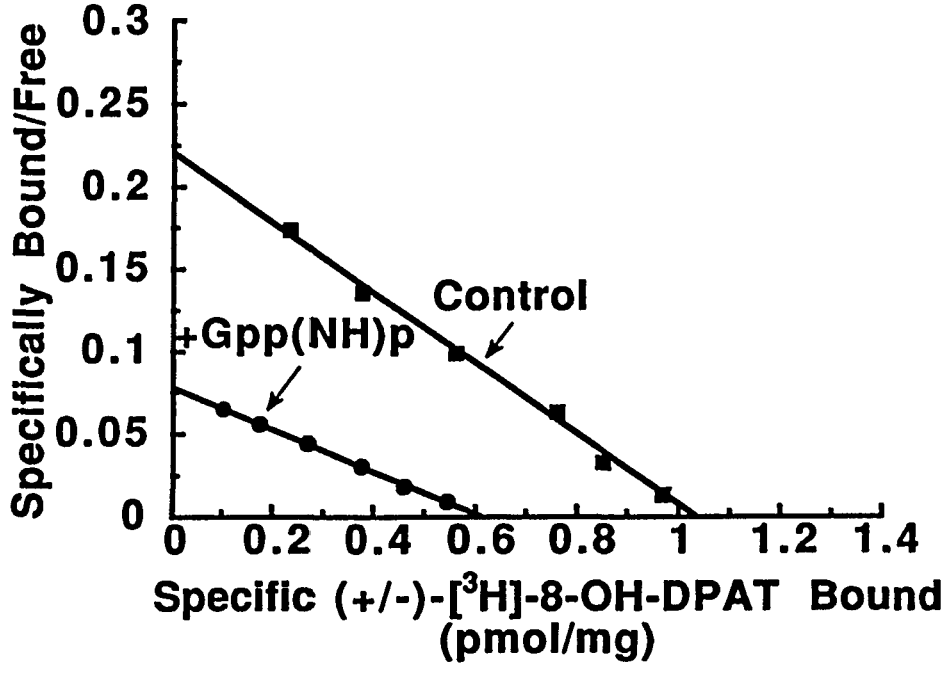
A. [^{3}H]-R-PIA


Control
+Gpp(NH)p
Non-Specific Binding
[^{3}H]-R-PIA Bound (pmol/mg protein)
1.4
1
0.6
0.2
0
0
1
2
3
4
5
[^{3}H]-R-PIA Free (nM)

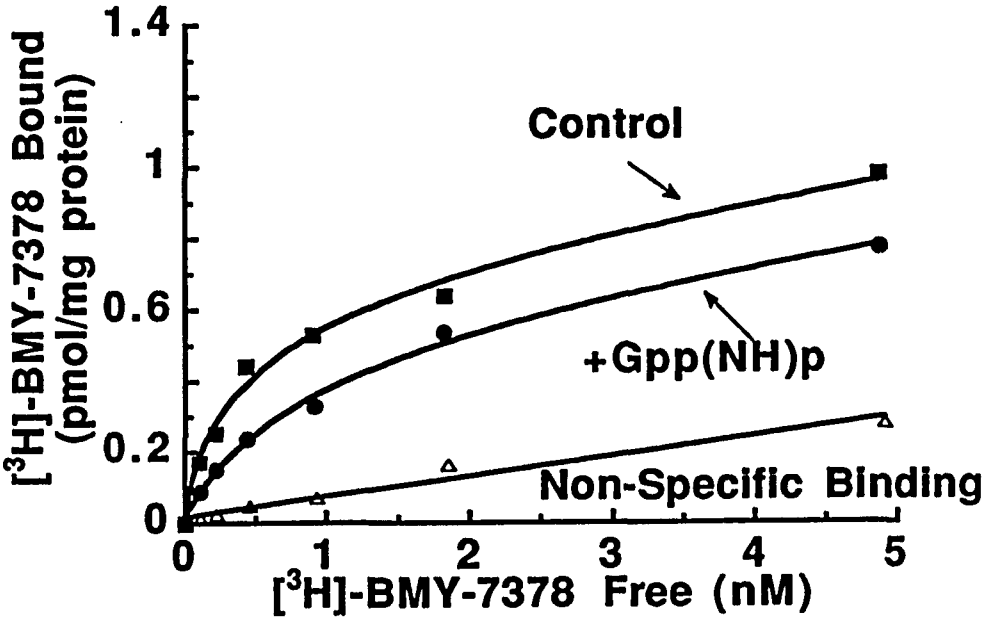

Control
+Gpp(NH)p
Specifically Bound/Free
0.3
0.2
0.1
0
0
0.2
0.6
1
1.4
Specific [^{3}H]-R-PIA Bound (pmol/mg)

B. (+/-)-[^{3}H]-8-OH-DPAT


(+/-)-[3H]-8-OH-DPAT Bound (pmol/mg protein)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
+Gpp(NH)p
Non-Specific Binding
0
1
2
3
4
5
(+/-)-[3H]-8-OH-DPAT Free (nM)


Specifically Bound/Free
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
+Gpp(NH)p
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Specific (+/-)-[3H]-8-OH-DPAT Bound (pmol/mg)

C. [^{3}H]-BMY 7378


[3H]-BMY-7378 Bound (pmol/mg protein)
1.4
1
0.6
0.2
0
Control
+Gpp(NH)p
Non-Specific Binding
0
1
2
3
4
5
[3H]-BMY-7378 Free (nM)

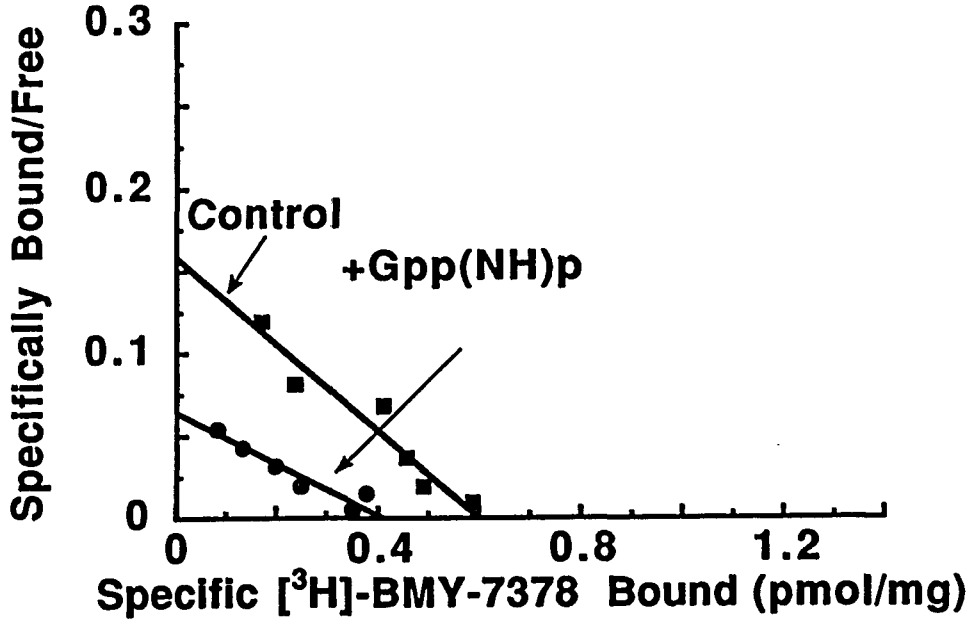

Specifically Bound/Free
0.3
0.2
0.1
0
Control
+Gpp(NH)p
0
0.4
0.8
1.2
Specific [3H]-BMY-7378 Bound (pmol/mg)

Fig. 4. Concentration-effect curves illustrating the relationship between Gpp(NH)p concentration and K_d and B_{max} for values for [^{3}H]-R-PIA (A), (±)-[^{3}H]-8-OH-DPAT (B) and [^{3}H]-BMY-7378 (C). Data points shown were calculated from six point concentration-binding curves in the presence of the indicated Gpp(NH)p concentration. Each data point reflects the mean ± SE of three experiments performed in duplicate. In each repetition, all [^{3}H]-agonists were tested in the same membrane preparation. The reduction in B_{max} occurring with progressive concentrations of Gpp(NH)p was fit to the logistic equation (Materials and Methods) generating the following parameters:

Parameter	[^{3}H]-R-PIA	(±)-[^{3}H]-8-OH-DPAT	[^{3}H]-BMY-7378
Basal B_{max} (pmol/mg)	1.56	1.05	0.62
E_{max} (pmol/mg)	0.75	0.51	0.39
E_{max} (%)	52	51	37
EC_{50}	1.30 µM	0.35 µM	0.47 µM
Slope index	1.09	0.74	0.61
Control K_d	0.35 ± 0.02	0.36 ± 0.04	0.40 ± 0.07

Control B_{max} values represent basal values, i.e., in the absence of Gpp(NH)p. E_{max} values can be expressed as pmol/mg protein or percent reduction of controls. The effect of Gpp(NH)p on K_d values was evaluated by correlation analysis, yielding ([^{3}H]-R-PIA, (±)-[^{3}H]-8-OH-DPAT, and [^{3}H]-BMY-7378 respectively), r = 0.0074, 0.313, and 0.258, indicating no dependence of K_d on Gpp(NH)p concentration.

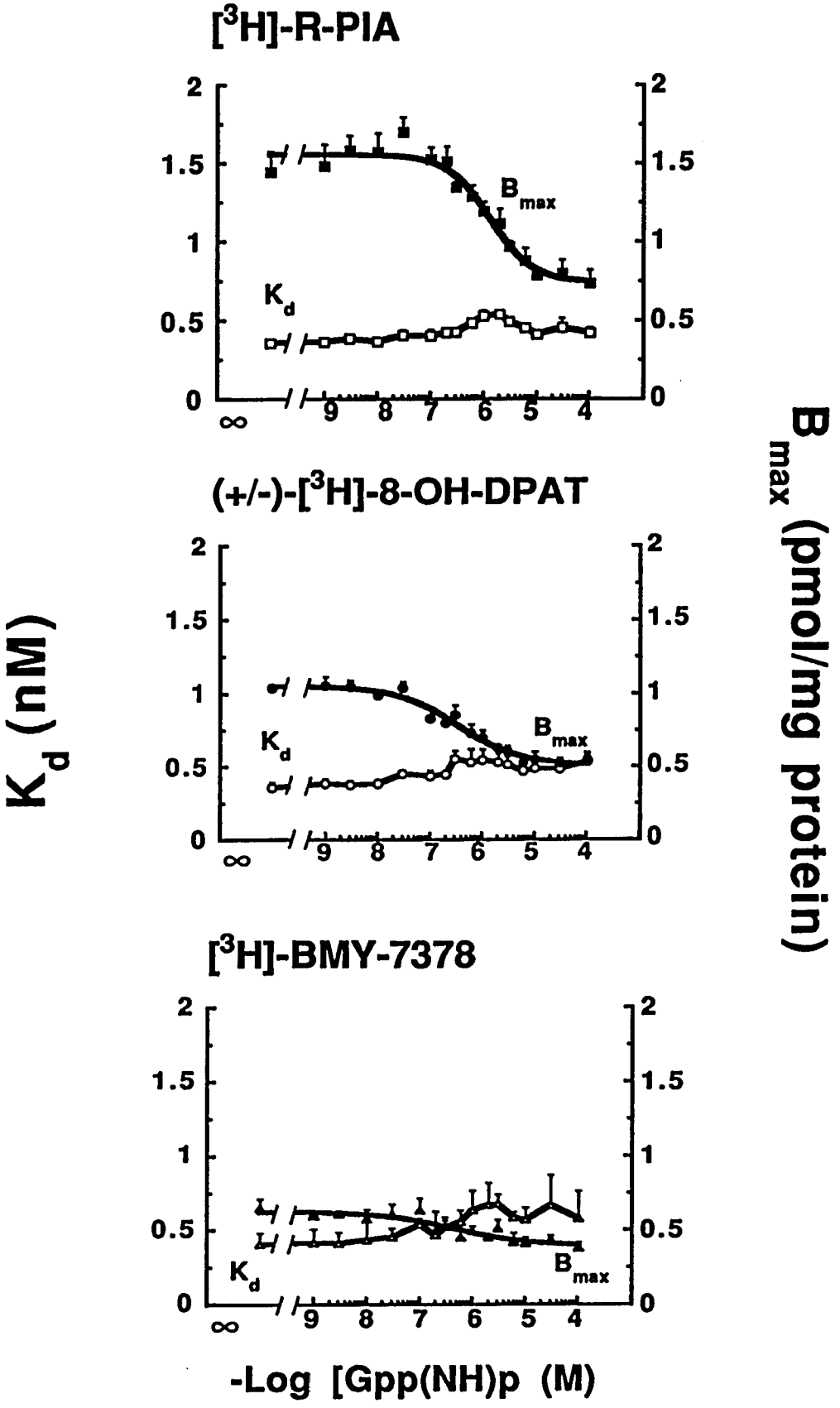

[3H]-R-PIA
(+/-)-[3H]-8-OH-DPAT
[3H]-BMY-7378
Kd (nM)
Bmax (pmol/mg protein)
Bmax
Kd
2
1.5
1
0.5
0
∞
9
8
7
6
5
4
-Log [Gpp(NH)p (M)

Fig. 5. Concentration-effect curves for the attenuation of [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT binding (both at 0.125 nM, or 24% occupancy) by Gpp(NH)p. Data are expressed as percent [^{3}H]-agonist bound in the absence of Gpp(NH)p. Each point represents the mean of three separate experiments ± SE, performed in duplicate. Each individual repetition for all [^{3}H]-agonists was performed in a single membrane preparation. Parameters of the concentration-effect curves fit to the logistic equation (Materials and Methods), are listed below. These values are similar to the mean of the three individual experiments (Table 2).

[^{3}H]-Agonist	E_{max} (% inhibition)	EC_{50} (μM)	slope index	Initial density (pmol/mg protein)
R-PIA	50	0.5	1.35	0.30 ± 0.01
(±)-8-OH-DPAT	58	0.13[a]	0.67[a]	0.23 ± 0.01

[a]Values statistically different ($p \leq 0.05$) from those obtained with [^{3}H]-R-PIA.

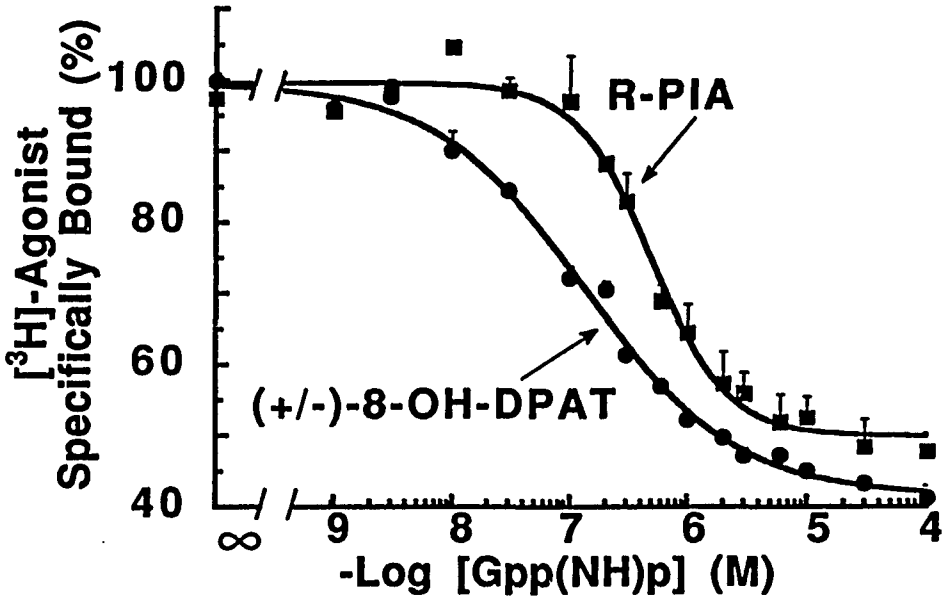

[3H]-Agonist Specifically Bound (%)
100
80
60
40
R-PIA
(+/-)-8-OH-DPAT
∞
9
8
7
6
5
4
-Log [Gpp(NH)p] (M)

TABLE 2

Parameters of Gpp(NH)p concentration-effect curves for the attenuation of [^{3}H]-agonist binding as a function of initial receptor occupancy by the [^{3}H]-agonist.

Means $\pm$ SE reflect values obtained from three separate experiments where the data were fit to the logistic equation (Materials and Methods) to obtain the parameters shown below. The values listed in parentheses in the pEC_{50} column represent the arithmetic mean of the EC_{50}. Each individual repetition for all three [^{3}H]-agonists was performed in the same membrane preparation.

Receptor Subtype & [^{3}H]-Agonist	% Initial Receptor Occupancy	pEC_{50} Gpp(NH)p Titration	E_{max} (%)	slope index
Adenosine A_1	24%	6.26 ± 0.09 (0.57 μM)	50 ± 3	1.39 ± 0.24
R-PIA	38%	6.28 ± 0.06 (0.53 μM)	51 ± 3	1.30 ± 0.38
	56%	6.17 ± 0.10 (0.70 μM)	51 ± 3	1.24 ± 0.19
	71%	6.04 ± 0.09 (0.93 μM)	52 ± 3	1.20 ± 0.18
	83%	5.83 ± 0.09[b] (1.51 μM)	52 ± 3	1.10 ± 0.18
	93%	5.60 ± 0.09[b] (2.58 μM)	48 ± 3	1.34 ± 0.27
5-HT_1A	24%	6.90 ± 0.09[a] (0.13 μM)	59 ± 1	0.67 ± 0.04[a]
(±)-8-OH-DPAT	38%	6.78 ± 0.03[a] (0.17 μM)	56 ± 1	0.80 ± 0.05
	56%	6.74 ± 0.15[a] (0.20 μM)	57 ± 3	0.63 ± 0.07[a]
	71%	6.66 ± 0.09[a] (0.23 μM)	55 ± 1	0.63 ± 0.18[a]
	83%	6.33 ± 0.04[a,b] (0.47 μM)	50 ± 3[d]	0.74 ± 0.03
	93%	6.13 ± 0.05[a,b] (0.75 μM)	50 ± 1[d]	0.63 ± 0.05[a]
5-HT_1A	24%	6.98 ± 0.27[a] (0.13 μM)	54 ± 4	0.54 ± 0.06[a]
BMY-7378	38%	6.83 ± 0.13[a] (0.15 μM)	50 ± 2	0.77 ± 0.10
	56%	6.80 ± 0.002[a] (0.16 μM)	52 ± 4	0.53 ± 0.12[a]
	71%	6.43 ± 0.08[a] (0.38 μM)	46 ± 1[c]	0.67 ± 0.02[a]
	83%	6.20 ± 0.25 (0.79 μM)	38 ± 5[d]	0.53 ± 0.19[a]
	93%	6.33 ± 0.39[a] (0.77 μM)	31 ± 4[c,d]	0.96 ± 0.09

a = Values (EC_{50} and slope index) significantly different from [^{3}H]-R-PIA at a similar level of occupancy ($p \leq 05$).

b = Values (EC_{50}) significantly different from lower occupancy levels labeled by the same [^{3}H]-agonist ($p \leq 0.01$).

c = Values (E_{max}) significantly different from (±)-[^{3}H]-8-OH-DPAT at a similar level of occupancy ($p \leq 0.05$).

d = Values (E_{max}) significantly different from lower occupancy levels labeled by the same [^{3}H]-agonist ($p \leq 0.05$).

Fig. 6. Attenuation of [^{3}H]-agonist binding by Gpp(NH)p at different initial levels of receptor occupancy by [^{3}H]-R-PIA (A), (±)-[^{3}H]-8-OH-DPAT (B), and [^{3}H]-BMY-7378 (C). Data were fit to the logistic equation (Material and Methods) and are expressed as percent of [^{3}H]-agonist binding in the absence of Gpp(NH)p. Points reflect mean ± SE for three individual experiments performed in duplicate. Each individual repetition for all three [^{3}H]-agonists was performed in a single membrane preparation. Mean ± SE for all six initial levels of occupancy are shown in Table 2, while parameters of the concentration effect curves shown above are as follows:

Initial Receptor Occupancy	Parameter	[^{3}H]-R-PIA	(±)-[^{3}H]-8-OH-DPAT	[^{3}H]-BMY-7378
24%	Initial Density (pmol/mg protein)	0.30 ± 0.01	0.23 ± 0.01	0.13 ± 0.03
	EC_{50} (μM)	0.50	0.135	0.10
	E_{max} (%)	50	58	54
	slope index	1.35	0.67	0.45
71%	Initial Density (pmol/mg protein)	1.08 ± 0.05	0.77 ± 0.02	0.44 ± 0.04
	EC_{50} (μM)	0.844	0.219	0.35
	E_{max} (%)	51	50	45
	slope index	1.17	0.64	0.68
93%	Initial Density (pmol/mg protein)	1.48 ± 0.06	1.11 ± 0.02	0.69 ± 0.04
	EC_{50} (μM)	2.17	0.736	0.72
	E_{max} (%)	48	49	30
	slope index	1.26	0.62	0.76

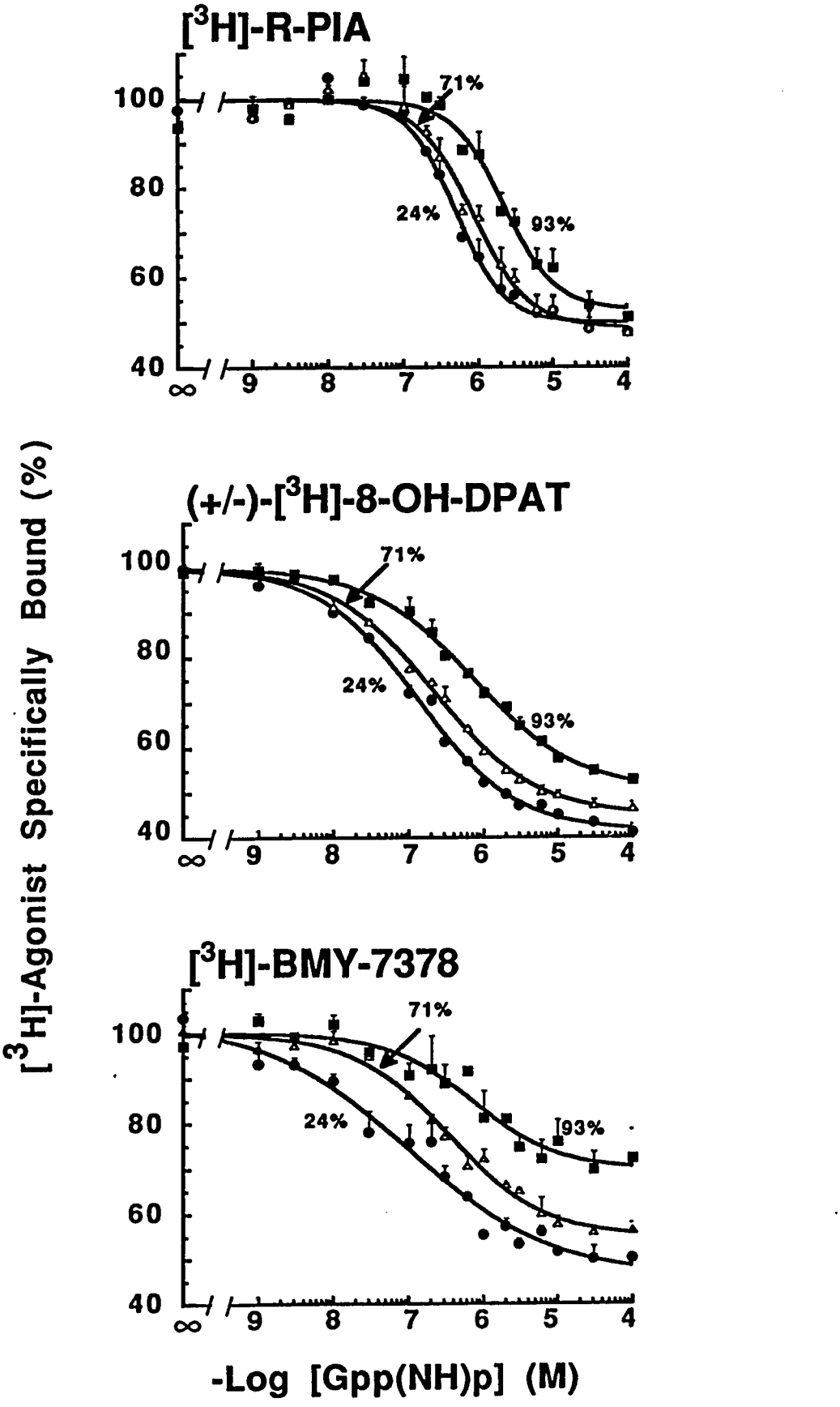

[3H]-R-PIA
71%
24%
93%
100
80
60
40
∞
9
8
7
6
5
4
(+/-)-[3H]-8-OH-DPAT
71%
24%
93%
[3H]-BMY-7378
71%
24%
93%
[3H]-Agonist Specifically Bound (%)
-Log [Gpp(NH)p] (M)

Fig. 7. Density of Gpp(NH)p-sensitive (100 μM) sites at each concentration of [^{3}H]-agonist. Data points shown represent the mean ± SE for three separate experiments. Points were calculated as percent of sites sensitive to Gpp(NH)p at each of the [^{3}H]-agonist concentrations shown in Fig. 5 and Table 2, but expressed as pmol/mg protein. Similar curves could also have been generated by direct subtraction of the specific binding curves shown in Fig. 3. Curves were computer fit to the Michaelis-Menten equation (Materials and Methods), which generated the following parameters:

[^{3}H]-Agonist	K_d (nM)	B_{max} (pmol/mg protein)
R-PIA	0.42	0.77
(±)-8-OH-DPAT	0.43	0.59
BMY 7378	0.22	0.21

The B_{max} for the density of sites sensitive to Gpp(NH)p is significantly ($p \leq 0.05$) greater for (±)-[^{3}H]-8-OH-DPAT when compared to [^{3}H]-BMY-7378, while the density at each concentration of all three [^{3}H]-agonists was significantly ($p \leq 0.01$) different from each other.

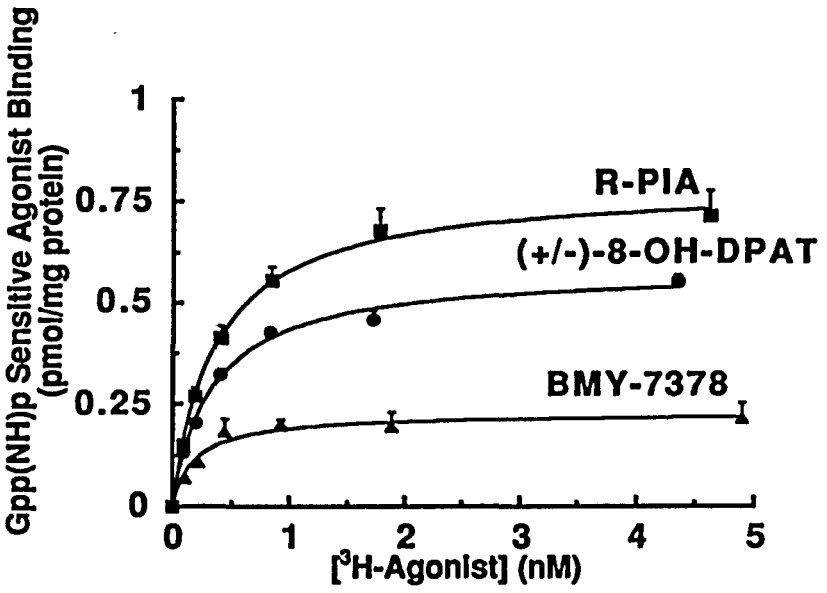

Gpp(NH)p Sensitive Agonist Binding
(pmol/mg protein)
1
0.75
0.5
0.25
0
0
1
2
3
4
5
[3H-Agonist] (nM)
R-PIA
(+/-)-8-OH-DPAT
BMY-7378

Fig. 8. Effect of [^{3}H]-agonist concentration (A) or receptor initial occupancy by the [^{3}H]-agonist (B) on IC_{50} of the Gpp(NH)p concentration-effect curve. Values shown represent the mean of three separate expériments ± SE. Approximate concentration of agonist (nM), 0.125, 0.250, 0.50, 1.0, 2.0, and 5.0 represents 24, 38, 56, 71 83, and 93% receptor occupancy, respectively. Data for [^{3}H]-agonist concentration versus Gpp(NH)p concentration were fit by linear regression followed by a two tailed correlation analysis ($p \leq 0.001$, [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT, and $p \leq 0.01$ [^{3}H]-BMY-7378. IC_{50} values for [^{3}H]-R-PIA were significantly different from (±)-[^{3}H]-8-OH-DPAT and [^{3}H]-BMY-7378, which were indistinguishable. * denotes IC_{50} values for [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT are significantly ($p \leq 0.01$) different from lower concentrations or levels of occupancy.

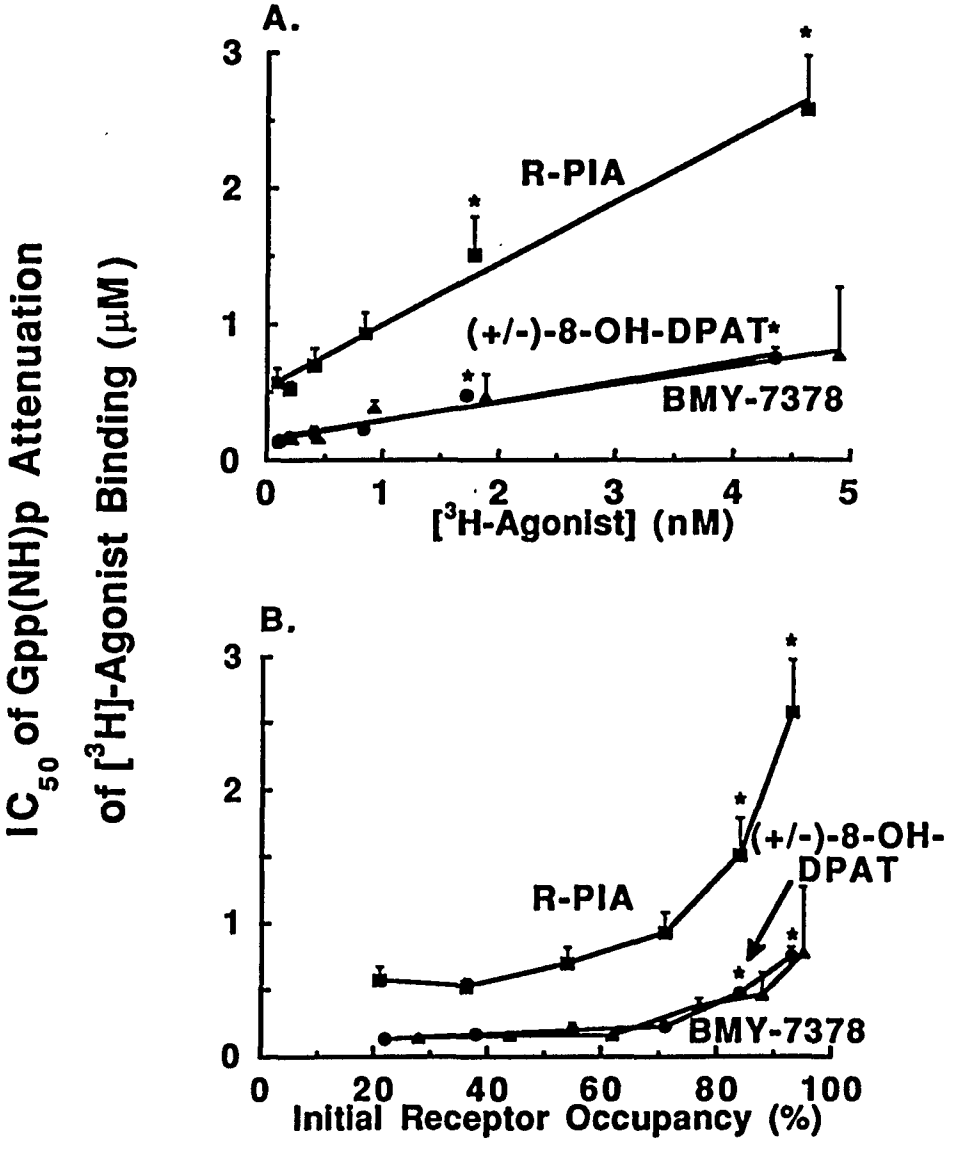

A.
B.
R-PIA
(+/-)-8-OH-DPAT
BMY-7378
[3H-Agonist] (nM)
Initial Receptor Occupancy (%)
IC50 of Gpp(NH)p Attenuation
of [3H]-Agonist Binding (μM)

TABLE 3

Parameters of GTPγS concentration-effect curves for the attenuation of [^{3}H]-agonist binding as a function of a progressive increase in the initial receptor occupancy by the [^{3}H]-agonist.

Means ± reflect values obtained from two separate experiments where the data were fit to the logistic equation (Materials and Methods) to obtain the parameters shown below. The values listed in parentheses in the pEC_{50} column represent the arithmetic mean of the EC_{50}. For any single repetition, all three agonists at all concentrations were performed in the same membrane preparation. This set of experiments was performed twice, thus no statistical evaluation was conducted.

Receptor Subtype & Agonist	% Initial Receptor Occupancy	pEC_{50} GTPγS Titration	Emax (%)	Slope	GTPγS Sensitivity (pmol/mg)
Adenosine A_1	24%	7.57 ± 0.21 (0.029 μM)	65 ± 6	0.85 ± 0.07	0.26 ± 0.08
[^{3}H]-R-PIA	38%	7.52 ± 0.07 (0.031 μM)	64 ± 4	0.94 ± 0.06	0.42 ± 0.10
	56%	7.42 ± 0.1 (0.038 μM)	63 ± 4	0.97 ± 0.09	0.59 ± 0.14
	71%	7.40 ± 0.16 (0.042 μM)	61 ± 4	1.09 ± 0.008	0.77 ± 0.19
	83%	7.33 ± 0.18 (0.049 μM)	60 ± 4	1.09 ± 0.07	0.90 ± 0.18
	93%	7.22 ± 0.09 (0.062 μM)	54 ± 2	1.06 ± 0.03	0.94 ± 0.21
5-HT_1A	24%	7.95 ± 0.12 (0.012 μM)	68 ± 4	0.80 ± 0.08	0.19 ± 0.09
(±)-[^{3}H]-8-OH-DPAT	38%	7.88 ± 0.25 (0.014 μM)	67 ± 4	0.73 ± 0.16	0.31 ± 0.14
	56%	7.72 ± 0.21 (0.020 μM)	63 ± 7	0.62 ± 0.10	0.42 ± 0.16
	71%	7.61 ± 0.19 (0.026 μM)	64 ± 4	0.64 ± 0.11	0.60 ± 0.19
	83%	7.52 ± 0.29 (0.034 μM)	58 ± 2	0.76 ± 0.09	0.62 ± 0.16
	93%	7.22 ± 0.10 (0.061 μM)	50 ± 1	0.90 ± 0.09	0.61 ± 0.13
5-HT_1A	24%	7.87 ± 0.27 (0.015 μM)	60 ± 3	0.85 ± 0.10	0.08 ± 0.03
[^{3}H]-BMY-7378	38%	7.78 ± 0.25 (0.018 μM)	59 ± 7	0.91 ± 0.06	0.13 ± 0.06
	56%	7.61 ± 0.39 (0.03 μM)	58 ± 3	0.87 ± 0.14	0.20 ± 0.07
	71%	7.67 ± 0.40 (0.026 μM)	58 ± 8	0.69 ± 0.08	0.26 ± 0.10
	83%	7.79 ± 0.43 (0.020 μM)	46 ± 9	0.87 ± 0.38	0.29 ± 0.14
	93%	7.62 ± 0.63 (0.037 μM)	40 ± 6	1.2 ± 0.56	0.30 ± 0.09

Fig 9. Concentration-effect curves of the GTPγS and Gpp(NH)p attenuation of 0.125 nM [^{3}H]-R-PIA (A) and (±)-[^{3}H]-8-OH-DPAT (B). Data were expressed as percent of specific [^{3}H]-agonist binding in the absence of guanine nucleotide. Each point reflects the average of either two (GTPγS) or three (Gpp(NH)p) separate experiments. Data were fit to the logistic equation (Materials and Methods), with parameters of the GTPγS concentration-effect curves as follows (Gpp(NH)p parameters are as shown in Fig. 4):

	[^{3}H]-R-PIA	(±)-[^{3}H]-8-OH-DPAT
IC_{50} (μM)	0.025	0.01
E_{max} (%)	64	68
slope index	0.85	0.80

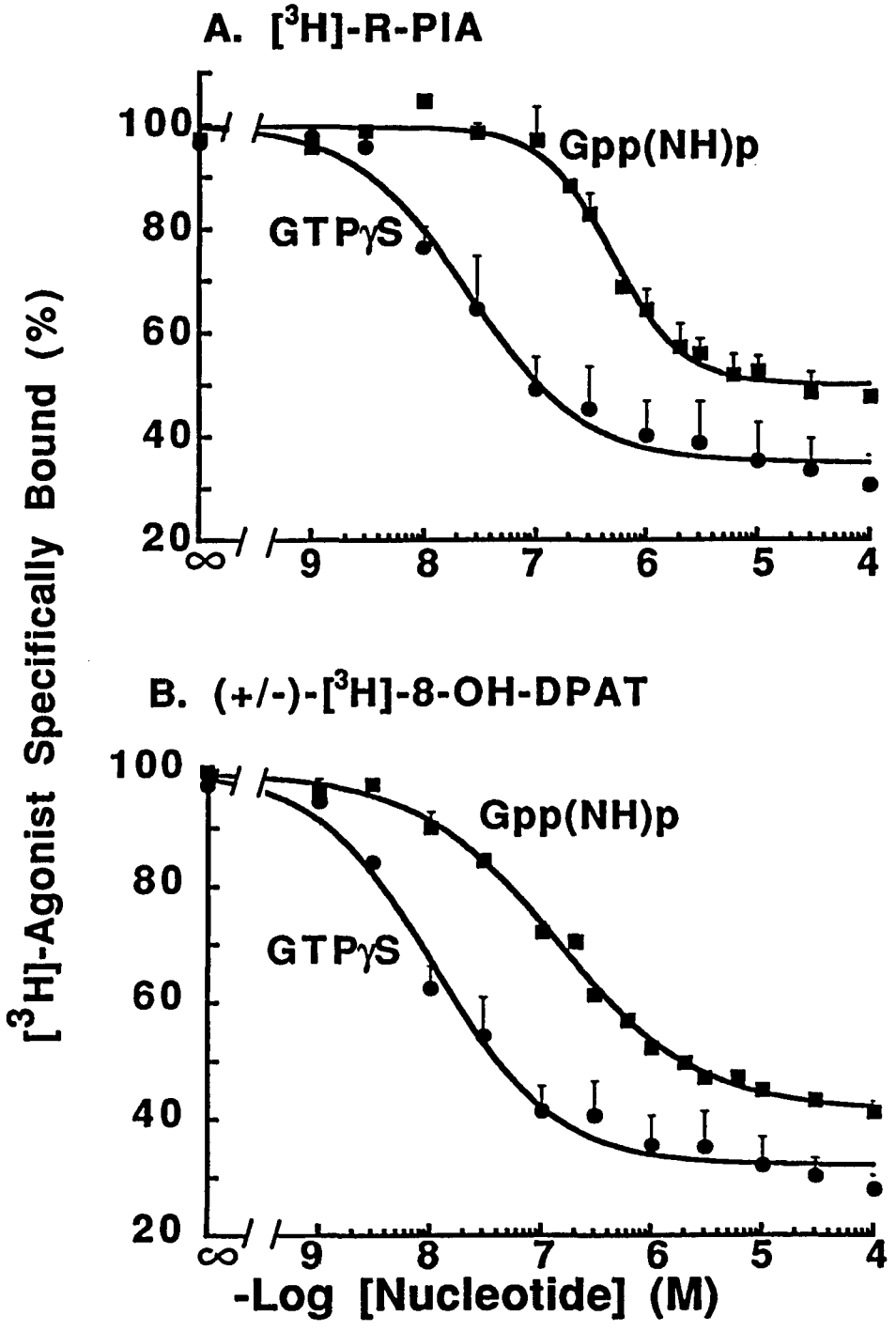

A. [3H]-R-PIA
100
80
60
40
20
∞
9
8
7
6
5
4
Gpp(NH)p
GTPγS
B. (+/-)-[3H]-8-OH-DPAT
100
80
60
40
20
∞
9
8
7
6
5
4
Gpp(NH)p
GTPγS
[3H]-Agonist Specifically Bound (%)
-Log [Nucleotide] (M)

Table 4

Some Characteristics of the Allosterism Between Binding of Drug and Guanine Nucleotide in Hippocampal Membrane Preparations.

Receptor System	Effect of Receptor Occupancy on Gpp(NH)p Attenuation of Agonist Binding	
	E_{max} Minor Changes	EC_{50} Large Increases
5-HT_{1A}	Approximately 50% at the lower occupancy levels, decreases as agonist concentration increases.	0.18--->0.8 (μM)
Adenosine A_1	Approximately 50%. No change over the entire range of agonist concentrations.	0.6--->2.6 (μM)

Receptor System	Effect of G-protein Occupancy by Gpp(NH)p on Agonist Binding Parameters	
	B_{max} Large Decreases	K_d (nM) Minor Changes
5-HT_{1A}	(±)-[^{3}H]-8-OH-DPAT 1.05--->0.51 (pmol/mg protein) [^{3}H]-BMY-7378 0.62--->0.39 (pmol/mg protein)	Both (±)-[^{3}H]-8-OH-DPAT and [^{3}H]-BMY-7378 in the absence of Gpp(NH)p are approximately 0.4 and are unchanged by the presence of guanine nucleotides.
Adenosine A_1	[^{3}H]-R-PIA 1.56--->0.75 (pmol/mg protein)	[^{3}H]-R-PIA, 0.4, and is unchanged by the presence of guanine nucleotides.

A Comparative Quantitative Autoradiographic Study of Adenosine A_1 and 5-Hydroxytryptamine$_{1A}$ Receptor Sites in Rat and Human Hippocampus

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Mount Sinai School of Medicine of the City University of New York
New York, New York 10029

Running Title: Adenosine A_1 and 5-HT_{1A} Receptors in Human Hippocampus

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Mt. Sinai School of Medicine
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1 Gustave L. Levy Place
New York, NY 10029
(212) 241-7251

ABSTRACT

Affinity values of neurotransmitters and drugs for membrane receptors, such as dissociation constants (K_d), are essential pharmacological tools for mutual classification of receptors and drugs. Measurement of K_d values in discrete regions of the brain can be done *in situ* by quantitative receptor autoradiography (QRA). In the present comparative QRA study, using both human and rat brain, we have examined two receptors, adenosine (AD) A_1 and 5-hydroxytryptamine (5-HT)$_{1A}$, in 9-13 hippocampal subregions. Complete concentration-binding curves were constructed, each with seven concentration points of either of two tritiated agonists: (±)-[^{3}H]-8-OH-DPAT (5-HT$_{1A}$) and [^{3}H]-R-PIA (AD A_1). The selectivity and high affinity of these drugs, coupled with the selectivity of the masking drugs (BMY-7378 and cyclopentyltheophylline) enabled a consistent generation of a high signal to noise ratio (9:1) even at the highest radioligand concentrations examined (10 nM).

(±)-[^{3}H]-8-OH-DPAT displayed similar affinity for the receptor sites in both species which was independent upon the tested region (K_d=0.4 nM). In contrast, the density (B_{max}; fmol/mg tissue) of 5-HT$_{1A}$ receptor sites varied across the subregions of the hippocampus: 22 to 192 in rat, and 51 to 276 in human. [^{3}H]-R-PIA displayed lower K_d values in rat (0.6-0.9 nM) than in human (1.0-1.7 nM) hippocampal subregions. The density of the AD A_1 receptor sites varied regionally (B_{max}; fmol/mg tissue), from 81 to 240 in rat, and from 108 to 240 in human hippocampus.

Compared to a membrane preparation from rat hippocampus, the K_d values of [^{3}H]-R-PIA (0.35±0.02 nM) and of (±)-[^{3}H]-DPAT (0.36±0.04 nM) were similar to values determined by QRA. If the assumption is made that the tissue sections are composed of 10% protein (METHODS), the fmol/mg tissue value can be converted to pmol/mg protein, enabling a comparison of density obtained autoradiographically with that obtained using conventional binding techniques. A difference in this parameter becomes readily apparent, where B_{max} values were 2-3 times higher than those obtained in the membrane preparation.

In this first comparative QRA study of two receptors in two species we demonstrate the validity of this method to measure *in situ* binding parameters of drugs to their respective receptors. These recognition-related parameters of drugs are crucial for comparative studies across species, tissues and drugs.

INTRODUCTION

Studies of receptors *in situ* by quantitative receptor autoradiography (QRA) have flourished during the last decade (Kuhar et al., 1986). This method has contributed valuable information to many aspects of neuroscience, such as receptor localization (Palacios et al., 1986), receptor modulation (Fastbom and Fredholm, 1990a; Welner et al., 1989; Frazer and Hensler, 1990; Radja et al., 1989) and receptor ontogeny (Shapiro and Insel, 1989; Palacios et al., 1988; Miyoshi and Kito, 1990). In general, once a selective drug is developed for a given receptor, it is a useful candidate for radiolabeling and use as a probe for QRA studied in the CNS and in the periphery. Two membrane receptors, the adenosine A_1 (AD A_1) (Fastbom et al., 1987a; Fastbom et al., 1987b) and the 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) (Marcinkiewicz et al., 1984; Kohler et al., 1986; Thor et al., 1990) are good examples of receptors in brain where the technique of QRA has been used for pharmacological identification and localization.

Pharmacological, biochemical and electrophysiological studies have revealed the existence of multiple subtypes of serotonin receptors (Bradley et al., 1986). QRA has revealed anatomic localization (Pazos et al., 1987a; Pazos et al., 1987b; Pazos and Palacios, 1985; Pazos et al., 1985), as well as phylogenetic differences (Martial, et al., 1989; Hoyer, et al., 1986). The heterogeneity in 5-HT_1 binding sites, each displaying a high affinity for [^{3}H]-5-HT (Pedigo et al., 1981; Heuring and Peroutka, 1987) makes the use of ligands to

block other subtypes, a necessity. (±)-[^{3}H]-8-OH-DPAT, a selective 5-HT_{1A} agonist (Hall et al., 1985), has been used to for QRA, to localize and quantitate 5-HT_{1A} receptors (Marcinkiewicz, et al., 1984; Kohler, et al., 1986). In contrast, based upon pharmacological and biochemical studies, adenosine receptors are divided into at least two subtypes (Van Calker et al., 1979). Potent and selective radioligands for the AD A_1 receptor are plentiful (Schwabe and Trost, 1980; Weber et al., 1990; Klotz et al., 1989; Snowhill and Williams, 1986). The most detailed characterization of these receptors has been done in rodent brain (Snowhill and Williams, 1986; Erfurth and Reddington, 1986). While the distribution of these receptors has been identified in human brain (Fastbom et al., 1987b; Kohler et al., 1986), a thorough quantitative characterization has not been reported.

We have reported on the coexistence of the 5-HT_{1A} and the AD A_1 receptors on pyramidal cells in rat hippocampus, and proposed that they may share some elements of signal transduction (Zgombick et al., 1989). In this study we have made a comparative QRA study of these receptors sites in human and rat hippocampus. We report on the distinct distribution of the two receptor sites across 9-13 subregions of the hippocampus of the two species, and compare binding parameters of the receptor sites in rat hippocampal membranes and sections.

MATERIALS AND METHODS

Tissue Preparation. Male Sprague Dawley rats (200-250 g, Charles River, Boston, MA) were asphyxiated using CO_2 and decapitated. The brain was removed from the skull, immediately frozen on dry ice and stored at -80°C until sectioned. Human brain tissue was obtained from the Department of Neuropathology at Mt. Sinai Medical Center (New York, NY). Seven brains (4 males and 3 females) with no prior history of neurological or psychiatric disease were used in this study. Tissue was obtained at autopsy (usually within 8 hours postmortem), and immediately frozen on dry ice, and stored at -80°C. A history of the human tissue used in the subsequent experiments is summarized in Table 1.

Brain tissue was mounted on a microtome chuck using Tissue-Tek embedding medium, and coronally sectioned, 15 μ thick, in a cryostat at -20°C. Sections were collected and thaw mounted on cold (-20°C) gelatin coated slides (0.5% gelatin, 0.05% chromium potassium phosphate). The sectioned tissue was dried under vacuum and stored at -20°C until use (generally less than one month). Prior to assay, slides were warmed to room temperature on a heating plate.

High Affinity Binding Assay. The *in vitro* binding of [^{3}H]-R-PIA, and (±)-[^{3}H]-8-OH-DPAT to tissue sections was performed by modification of the method of Pazos et al. (Pazos et al., 1988). Briefly, slides were preincubated at room temperature for 15 min. in

assay buffer, (50 mM HEPES, 0.1% ascorbate, 2 mM EGTA, 2.5 mM $MgCl_2$, pH adjusted to 7.4 at room temperature with saturated Tris base). For [^{3}H]-R-PIA binding, 2 IU/ml of adenosine deaminase was added, to remove endogenous adenosine (Linden, 1989).

Slide mounted tissue sections (1-2 sections/slide) were incubated at room temperature in 40 ml of either [^{3}H]-R-PIA (36-42 Ci/mmol) or (±)-[^{3}H]-8-OH-DPAT (125-142 Ci/mmol), or the radioligand plus the respective masking ligand diluted in assay buffer. Preliminary kinetic studies have demonstrated the association of agonist to receptor to approach equilibrium within 60 mins. (data not shown), therefore, incubation of tissue slices with radioactive drug, were conducted for 60 mins. For saturation experiments, the concentrations of radioligand was varied from 0.1 nM to 10 nM. Non-specific binding was defined by 10 µM CPT ([^{3}H]-R-PIA binding), or 1 µM BMY-7378 ((±)-[^{3}H]-8-OH-DPAT binding). Specific binding was 85-95% of total binding. After 60 mins. incubation in the respective radioisotope, the slides were washed two times, 5 mins. each, in ice cold assay buffer (4^{o}C), rinsed in ice-cold distilled water, and dried under a stream of cold dry air.

Quantitative Receptor Autoradiography. Autoradiograms were generated by exposure of dried tissue slices to tritium sensitive film (HYPERFILM, Amersham, Arlington Heights, IL) at 4^o C, for 20 to 30 days. Quantitation of bound radioactivity, was done by coexposure of the slides with tritium standards embedded in a polymer block of methacrylate (AMERSHAM, [^{3}H]-microscales). Films were developed

using Kodak D-19 developer, fixed in Kodak Rapid Fixer, rinsed, and allowed to dry at room temperature.

Data Analysis. Optical measurements were transferred to a MacIntosh II computer using a Cohu videocamera (San Diego, CA), Data Translation (Marlboro, MA) frame grabber board and Chromopro 45 (Tempe, AZ) light box. Conversion of optical density into density of ligand binding sites was done using IMAGE 1.33, the public domain program for digital image processing and analysis (Wayne Rasband, NIH, Bethesda, MD). Optical density measurements were converted to either DPM/mg tissue or fmol/mg tissue, using the [^{3}H]-microscales (Fig. 1a) which contain conversions for tissue equivalents (Geary, et al., 1985). Fig. 1b illustrates a plot of radioactivity concentration (fmol/mg tissue) against film optical density. While the lower concentrations of radioactivity produce a linear increase in optical density, higher concentrations of radioactivity cause the film to saturate. This curve is best approximated by a four parameter logistic equation (David Rodbard, NIH, Bethesda, MD and Cary Mariash, Univ. of Minnesota):

$$y = D + \frac{A-D}{1+(X/C)^B}$$

Where, the parameters are represented by:

A = Film Background

B = Slope Index

C = Midpoint of the curve

D = Maximal density

X = The concentration of radioactivity

The final values are obtained from the standard curve in fmol/mg tissue or dpm/mg tissue. In order to correlate these values to those previously reported in membrane preparations (CHAPTER 1), the assumption was made that while normal cells are composed of 3% particulate protein, the dried tissue sections are composed of up to 10% particulate protein (Frazer, personal communication). Non-specific binding was subtracted from total binding to generate specific binding. Identification of the brain regions as well as specific cell layers was made with the aid of cresyl violet stained sections, and identified according to standard neuroanatomical atlases (Duvernoy, 1988; Paxinos and Watson, 1986; DeArmond et al., 1976; and Barr and Kiernan, 1983).

Saturation binding data were analyzed using the nonlinear regression analysis program LIGAND (Munson and Rodbard, 1980). Binding curves were best fit to a one site model yielding K_d, B_{max} and Hill Coefficient. Statistical evaluation was done using one way

ANOVA, followed by Duncan's multiple range test. The accepted level of significance was $p \leq 0.05$.

Materials. (±)-[^{3}H]-8-OH-DPAT (125-140 Ci/mmol) and [^{3}H]-R-PIA (36-42 Ci/mmol) were purchased from New England Nuclear (Boston, MA.) CPT was purchased from Research Biochemicals, Inc. (Natick, MA). BMY-7378 was a generous gift from Dr. Frank Yocca of Bristol-Myers Squibb Co. (Wallingford, CT). All other chemicals used in assay buffer and in gelatin coating the slides, were purchased from Sigma Chemical Co. (St. Louis, MO), and were of the highest grade available.

RESULTS

Distribution of AD A_1 and 5-HT_{1A} Receptor Sites in Human and Rat Hippocampus. Figures 2 and 3 illustrate images generated on tritium-sensitive film depicting the binding of 2 nM [^{3}H]-R-PIA in the absence and presence of 10 μM CPT, to sections of human and rat hippocampus. To enable direct comparison with a study that we have previously performed in membrane preparations (CHAPTER 1), the present study was performed under identical assay conditions (Materials and Methods). This is the first quantitative autoradiographic study with the selective AD A_1 agonist R-PIA, the results of which demonstrate a large ratio of total to non-specific binding. Thus, the selected assay conditions coupled with this drug combination appears to be optimal for a comparative quantitative study across brain regions and species.

In both species, the CPT-sensitive [^{3}H]-R-PIA binding sites throughout the hippocampus, were distributed in a pattern similar to that previously reported using [^{3}H]-N^6-cyclohexyladenosine in rat (Goodman and Snyder, 1982, Fastbom et al., 1987a; Onodera and Kogure, 1988) and in human (Fastbom et al., 1987b) brain. However, the B_{max} values reported here are somewhat greater than previous reports.

As can be seen in Figs. 2 and 3, there are distinct differences between human and rat hippocampus. For example, in human, the highest density (fmol/mg tissue) appears to be in CA2 stratum

pyramidale and radiatum and CA3 stratum radiatum (approximately 120) while in rat the highest density was observed in CA1 stratum radiatum (approximately 300). A comparative study across the two species was done by constructing concentration-binding curves in several subregions of human and rat hippocampus (see below).

Figs. 4 and 5 illustrate the images produced on tritium-sensitive film from the binding of 2 nM (±)-[^{3}H]-8-OH-DPAT to sections of human and rat hippocampus in the absence and in the presence of 1 μM BMY 7378. Similar to the studies with [^{3}H]-R-PIA, the assay conditions were adopted from our studies done in membrane preparations and, with the exception of the presence of adenosine deaminase in the [^{3}H]-R-PIA assay, were identical to those utilized to study the AD A_1 sites. BMY-7378 was selected as a masking ligand for the determination of the "non-specific" binding, as this buspirone analogue displays a high selectivity for the 5-HT_{1A} receptor (Yocca, et al., 1987). As shown in Figs. 4 and 5, the signal to noise ratio in both species makes the assay conditions and the drug combination suitable for quantitative receptor autoradiography.

In both species, 5-HT_{1A} receptor sites were distributed in the hippocampal subregions in a pattern similar to that previously reported using [^{3}H]-5-HT (Pazos et al., 1987a; Pazos and Palacios, 1985), (±)-[^{3}H]-8-OH-DPAT (Marcinkiewicz et al., 1984), and [^{3}H]-ipsapirone (Glaser et al., 1985). In human, (±)-[^{3}H]-8-OH-DPAT displayed a high density of binding (fmol/mg tissue) in the entire CA1 region (120-210), moderate binding in the dentate gyrus (30-

60), and little binding in the CA3 or CA4 region. Similar results were observed in the rat, where (±)-[^{3}H]-8-OH-DPAT labels a very high density of sites in the entire CA1 region with CA1 stratum oriens, radiatum and lacunosum-moleculare displaying the highest densities (90-150 fmol/mg tissue). Also in agreement with the human hippocampus, is the absence of binding in the CA2 region. In contrast to human hippocampus, where the dentate gyrus displays only moderate binding, this region in the rat displayed the greatest density (150-180 fmol/mg tissue).

Because most sections of human hippocampus obtained for this study included a portion of cortex (e.g., Fig. 2, 4) we have made initial observations on the binding of these two ligands in this brain region. Unlike [^{3}H]-R-PIA, the binding of (±)-[^{3}H]-8-OH-DPAT in the human cortex appears to be unevenly distributed, with the highest binding occurring in the outer layers (90-120), moderate binding in the inner layers (30-60), and very little binding in middle layers. [^{3}H]-R-PIA binding is similar in most layers of the cortex.

Saturation analysis of [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT Receptor Sites in Rat and Human Hippocampi. To enable a complete quantitative regional comparison, we have characterized the binding of [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT by saturation analysis, using seven concentrations of the tritiated agonist per binding curve. Illustrated in Fig. 6 are typical results in one brain region (dentate gyrus) displaying "total" and "non-specific" binding, as well as Rosenthal transformations. Binding of both drugs in both

species was saturable and high affinity, displaying similar K_d values (nM, of illustrated curve (Fig. 6); and range of 2-6 repetitions). [^{3}H]-R-PIA: human (0.51; 0.5-2.38) and rat (0.68; 0.68-0.84), (±)-[^{3}H]-8-OH-DPAT: human (0.44; 0.2-0.65) and rat (0.30; 0.19-0.39). The density (B_{max}) of AD A_1 receptor sites (fmol/mg tissue, of shown curve; range of 2-6 repetitions) was also similar: human (122; 120-237) and rat (201; 201-246). In contrast, the density of (±)-[^{3}H]-8-OH-DPAT binding was higher in rat, (190; 177-216) than human dentate (97; 54-105).

A summary of binding parameters in 8-13 subregions of human and rat hippocampus is presented in tables 2-5. These data demonstrate that the K_d values appear to be relatively constant across the regions examined (ANOVA $p \geq 0.05$, [^{3}H]-R-PIA $F = 0.51$, 0.89 and (±)-[^{3}H]-DPAT $F = 0.95$ and 1.49, human and rat respectively). In contrast, B_{max} values were region dependent with statistical differences occurring between most of the subregions. Between species, (±)-[^{3}H]-DPAT affinity is remarkably consistent (nM; 0.4-0.6, human, and 0.3-0.6, rat). In contrast, [^{3}H]-R-PIA affinity was species dependent, with K_d values slightly higher in most subregions of human hippocampus (1.0-1.74 nM), compared to rat hippocampus (0.60-0.90 nM) (2 way ANOVA, with species as factor A and region as factor B, showed a statistical difference between species $F:(1,46) = 9.15$, $p<0.005$; no statistical difference was observed across regions $F:(9,46) = 0.55$, $p<0.83$). Variables (K_d, B_{max} and Hill coefficients) from the complete saturation analysis of [^{3}H]-R-

PIA and (±)-[^{3}H]-DPAT in rat and post-mortem human hippocampus are listed in Tables 2 to 5.

DISCUSSION

Hormone binding to a membrane receptor is the first event in the initiation of signal transduction mediated by the receptor. In the absence of multiple subtypes of a given receptor, the intrinsic properties of hormone binding, receptor density and hormone affinity, may contribute to observed differential responsiveness of a hormone across tissues, such as brain regions. Receptor autoradiography is a useful technique for the analysis of receptor distribution patterns across tissue (Kuhar et al., 1986). While many studies have presented accurate data dealing with the qualitative aspects of receptor distribution, the goal of the present study was to utilize the method of quantitative receptor autoradiography to measure the agonist-related binding parameters of density and affinity. Using two different agonists ([^{3}H]-R-PIA, an AD A_1, and (±)-[^{3}H]-8-OH-DPAT, a 5-HT_{1A} agonist), in two species (human and rat), we have evaluated binding parameters across several subregions of the hippocampus.

Studies of receptor density using a single concentration of [^{3}H]-agonist, across regions or groups, must be done at equal occupancy, which in turn can only be achieved by knowledge of the drug dissociation constant. Traditionally these parameters are evaluated from concentration-binding curves (saturation experiments). Due to the low ratio of specific to non-specific binding of many radioligands, and because optimal conditions for these studies should be worked

out specifically for the radioligand of choice, a key point in these studies is the identification of the preferred drug combination.

The combination of masking ligand and the [^{3}H]-agonist (CPT/[^{3}H]-R-PIA and BMY-7378/(±)-[^{3}H]-8-OH-DPAT) was proven to be optimal in this study. As can be seen in Figs. 2-5, and 6, very low levels of "non-specific" binding were observed with the two [^{3}H]-agonists in both species. Moreover, the same ratio of specific to non-specific binding was observed across all the tested tissues. Thus, the quantitative receptor autoradiographic method utilized in this study appears to provide accurate estimates of the binding parameters we proposed to evaluate. Complete concentration-binding curves were constructed using [^{3}H]-R-PIA in 11 regions of human hippocampus (Table 2) and 16 regions of rat brain (Table 3). Similar curves were constructed using (±)-[^{3}H]-8-OH-DPAT in 8 regions of human hippocampus (table 4) and 11 regions of rat brain (table 5).

The most striking observation is that the dissociation constant of each of the two drugs is independent of region (Tables 2-5). This is in accordance with a prior studies using [^{3}H]-cyclohexyladenosine, to autoradiographically quantitate the rat AD A_1 receptor in different brain regions (Snowhill and Williams, 1986; Erfurth and Reddington, 1986). In contrast, the density of the receptor sites varied dramatically across the tested regions: density (fmol/mg tissue) of the CPT-sensitive [^{3}H]-R-PIA binding sites ranged between 108 to 240 in human hippocampus, and between 81 to 255 in the rat brain. Similarly, the density of BMY-7378-sensitive (±)-[^{3}H]-8-OH-DPAT

binding sites ranged from 51 to 276 in human hippocampus, and 22 to 192 in rat brain.

Using rat hippocampal membrane homogenates, we have previously reported the binding parameters of [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT (CHAPTER 1). In whole hippocampal membrane preparations, [^{3}H]-agonist K_d values (nM) were: [^{3}H]-R-PIA, 0.35 ± 0.02 and (±)-[^{3}H]-8-OH-DPAT, 0.36 ± 0.04, while B_{max} values (pmol/mg protein) were: [^{3}H]-R-PIA, 1.44 ± 0.16, and [^{3}H]-8-OH-DPAT 1.04 ± 0.03. Using quantitative receptor autoradiography to measure subregions of rat hippocampus, yielded similar affinity parameters; [^{3}H]-R-PIA K_d values (nM) ranged in the various subregions from 0.6 to 1.37, and for (±)-[^{3}H]-8-OH-DPAT, from 0.2 to 0.6. However, for both receptors, the density was substantially higher in the autoradiographic study, as opposed to membrane preparations. Using autoradiography to analyze densities in subregions of hippocampus, produced B_{max} values (pmol/mg protein, METHODS) up to three to five times higher for [^{3}H]-R-PIA (0.85 to 3.6, Table 3) and (±)-[^{3}H]-8-OH-DPAT (0.2 to 1.7). This discrepancy in the B_{max} values may be due to the ability to analyze discrete regions displaying the highest densities, using quantitative receptor autoradiography, as opposed to a mixture of regions in membrane homogenates. The actual polytron homogenization of the tissue necessary to prepare membranes may cause a loss of receptors (Norman et al., 1989).

The increased density of receptors seen with QRA has been reported earlier (Erfurth and Reddington, 1986) with AD A_1 receptors. Similar incongruencies in B_{max} values between the two preparations have been reported in other receptor systems. Using [^{3}H]-QNB to measure the density of muscarinic receptors in hypothalamic nuclei, Rainbow et al. (Rainbow et al., 1984a) has reported a 2-3 times greater B_{max} value autoradiographically, than in the corresponding region using a membrane preparation. They also report a similar observation (Rainbow et al., 1984b) using [^{125}I]-pindolol to measure beta-adrenergic receptors in cerebral cortex. In the AD A_1 receptor system, using [^{3}H]-cyclohexyladenosine, B_{max} values up to 200 fmol/mg tissue have been reported (Snowhill and Williams, 1986). These are similar to those we obtained using autoradiography, and it one assumes 10% particulate protein, this produces B_{max} values for the AD A_1 receptor sites slightly higher than we obtained in membrane preparations.

Species differences have been reported in a variety of receptor systems including the beta-adrenergic (Booze et al., 1989), alpha2-adrenergic (Feller and Bylund, 1984), adenosine A_1 (Murphy and Snyder, 1982; Ferkany et al., 1986), and A_2 (Stone et al., 1988), and serotonergic (Hoyer and Middlemiss, 1989; Heuring, et al., 1986). A subtype of 5-$HT_{1\text{-like}}$ receptor, the 5-HT_{1D} (Heuring and Peroutka, 1987), has been found to occur in human, but not rodent brain (Schoeffter and Hoyer, 1989; Waeber et al., 1988; Waeber et al., 1989; Waeber et al., 1990). The apparent variability in receptors between species makes the choice of an appropriate human model

for preclinical evaluation of pharmacological agents extremely important.

In our autoradiographic comparison between rat and human hippocampus, while subtle differences occurred in the distribution of 5-HT_{1A} receptors, i.e., dentate gyrus and stratum pyramidale (Tables 4 and 5), B_{max} values (fmol/mg tissue, range) were quite consistent between the species (50-278, in human and 18-170 in rat). The affinity of the 5-HT_{1A} receptor for (±)-[^{3}H]-8-OH-DPAT was remarkably similar (K_d, nM, 0.4-0.5, human, and 0.3-0.6, rat). Corresponding to these results, it has been shown that (±)-[^{3}H]-8-OH-DPAT labels a single population of binding sites with a similar affinity in membrane preparations of rat, pig, and human cortex and hippocampus (Palacios et al., 1987a). Competition curves revealed the rank order of potency for more than a dozen drugs to be indistinguishable in rat, pig and human membranes. After surveying the 5-HT_{1A} receptor in numerous species (Pazos et al., 1988), pharmacology and distribution were determined to be well preserved, from fish to human. Therefore, phylogenetically, the 5-HT_{1A} receptor has been highly conserved, and the choice of an animal model is not of great concern.

While between species, density variations in AD A_1 receptors are minor (Ferkany,et al., 1986, Lee et al., 1986), many groups have reported changes in receptor affinity constants among species (Ferkany et al., 1986; Murphy and Snyder, 1982; and Schwabe et al., 1985). While regional differences were not statistically significant

between species, there was an overall statistical difference between affinity constants in human hippocampus (1.0-1.7) versus those in rat hippocampus (0.6-0.9 nM). Since the AD A_1 receptor has not been cloned in these species, one can only postulate that the differing affinity constants represent different receptors. Photoaffinity labeling of the AD A_1 receptor from hamster fat cells and rat brain, followed by carbohydrate analysis has yielded a similar molecular weight receptor, that is glycosylated differently (Klotz and Lohse, 1986). These studies point to the importance of consideration in the choice of an animal model for human pharmacology.

Both receptors chosen in this study displayed very high densities in the hippocampal region of both human and rat brain. Interestingly the AD A_1 and 5-HT_{1A} receptors are linked to similar effector mechanisms; inhibition of adenylyl cyclase activity (De Vivo and Maayani, 1986, Ebersolt et al., 1983) and membrane hyperpolarization, due to opening of potassium channels (Trussell and Jackson, 1987; Andrade et al., 1986). Common distribution and effectors, as well as the non-additivity in the maximal responses and the fact that *in vivo* treatment of rats with pertussis toxin, caused a similar decrease in the maximal responses, led Zgombick et al. (Zgombick, et al., 1989) to propose that these two receptors share a common pool of G-proteins.

In a previous report (CHAPTER 1), we have probed the allosterism existing between the binding of hormone to receptor and guanine nucleotide to G-protein(s). In an effort to move this work

from membrane preparations to coronal sections of tissue, where regional heterogeneity can be examined, it was necessary to first characterize the binding characteristics of AD A_1 and 5-HT_{1A} receptors. In summary, the method of quantitative autoradiography enabled us localize and quantitate with respect to density and affinity, parameters related to hormone interaction with receptor, for two different receptor systems (AD A_1 and 5-HT_{1A}), in two different species (rat and human). In future publications we will address the allosterism between the binding of hormone to receptor and guanine nucleotide to G-protein(s), in the autoradiographic preparation. This is an important step toward the ultimate integration of the initial events of signal transduction with respect to the anatomy of the brain.

Table 1

History of Human Tissue Used for Quantitative Receptor Autoradiography.

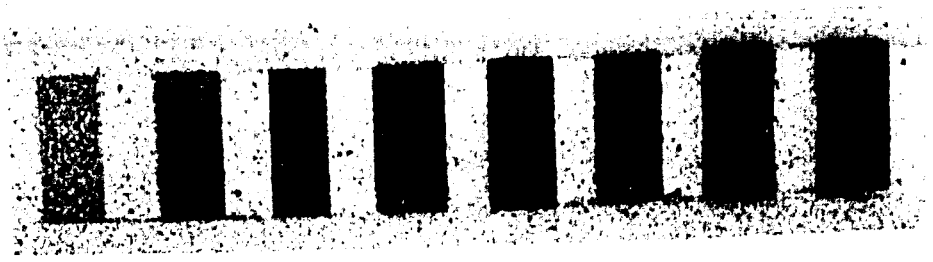
Tissue was obtained from The Mount Sinai School of Medicine, Department of Pathology, (New York, NY), and immediately frozen on dry ice, prior to storage at -80°C. Tissue was prepared for quantitative receptor autoradiography as described in Materials and Methods.

Sample #	Sex	Age (yrs.)	Post-mortem delay (hrs)	Pre-mortem condition	Immediate cause of death	Date of death
1	M	76	6.0	Leukemia	Pneu-monia	5/19/89
2	M	79	5.0	Asthma	Hypoxia	8/24/89
3	F	62	6.0	None	Myo-cardial infarction	8/25/89
4	F	67	11.0	None	Myo-cardial infarction	1/26/90
5	F	88	12.0	Bladder carcinoma	Septic Shock	5/14/90
6	F	78	14.5	Chronic Bronchitis	Bronchitis	6/11/90
7	M	73	8.0	Chronic myelogenous leukemia	Congestive Heart Failure	12/21/90

Fig. 1. A representative calibration curve for quantitative autoradiography of receptor sites. A). Tritium standards ([^{3}H]-microscales) used for generation of the calibration curve. Different tritium concentrations are embedded in a block of methacrylate polymer (Amersham, Arlington Heights, IL). For a given experimental film, two sets of standards were co-exposed with tissue sections prepared for autoradiography (Materials and Methods). The illustrated standards represent a 27 day exposure time, where [^{3}H]-microscale values are compatible with exposure times of 1-8 weeks. The optical densities displayed represent tissue equivalents of 1.4, 2.2, 3.2, 5.5, 9.1, 13.2, 22.4, and 32.0 nCi/mg wet weight tissue (Vendors values). These values were transformed into either DPM/mg tissue or fmol/mg tissue using the radioligand specific activity (Ci/mmol), accounting for the tritium decay of the [^{3}H]-microscales. Either of these values of radioactive density is plotted against optical density. B). A standard curve produced from the images shown in A. The optical density values (0-250) were defined by the IMAGE program used on the Macintosh II computer. The background values of this film (A; see equation below) was 63 and the highest density was 240 (D). Tissue equivalent values range from 1.4 to 32 nCi/mg tissue. The collected values of optical density (Y) and the calculated density values of the radioactivity (X) were fit to the following equation (METHODS):

$$y = D + \frac{A-D}{1+(X/C)^B}$$

Where, A = 62.55, B = 1.307, C = 28.15, and D = 248.09, and r^2 = 0.9987.



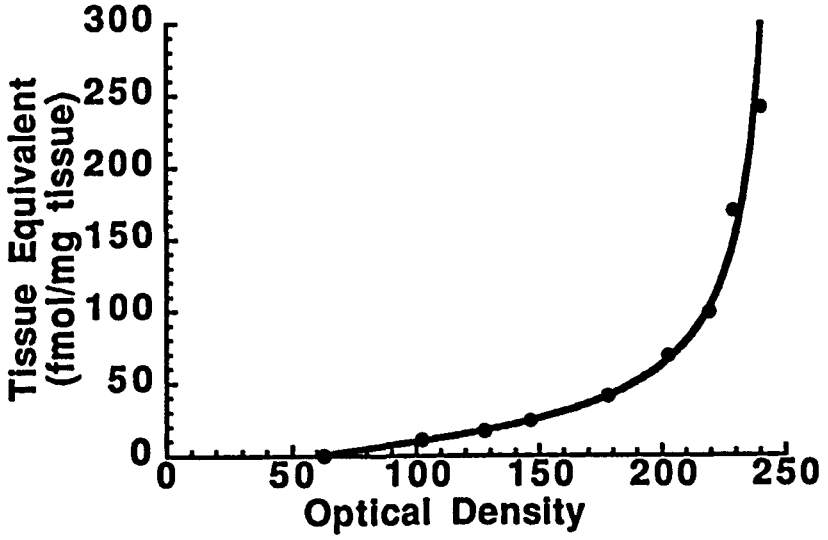

Tissue Equivalent
(fmol/mg tissue)
300
250
200
150
100
50
0
0
50
100
150
200
250
Optical Density

Fig. 2. Illustration of images produced on tritium-sensitive film by [^{3}H]-R-PIA (2 nM) bound to human hippocampal sections in the absence and the presence of 10 μM CPT Sections (15 μ) of 6 hr. post-mortem human hippocampus (subject #3, Table 1) were prepared for quantitative receptor autoradiography as described in Materials and Methods, and exposed to HYPERFILM for 43 days. The binding of [^{3}H]-R-PIA in the presence of CPT ("non-specific") was not significantly different from film background. Binding at the level of CA1 stratum pyramidale (198 fmol/mg tissue), and dentate gyrus (141 fmol/mg tissue) was similar in another 5 samples and in proportion to the B_{max} values of these regions (Table 2).

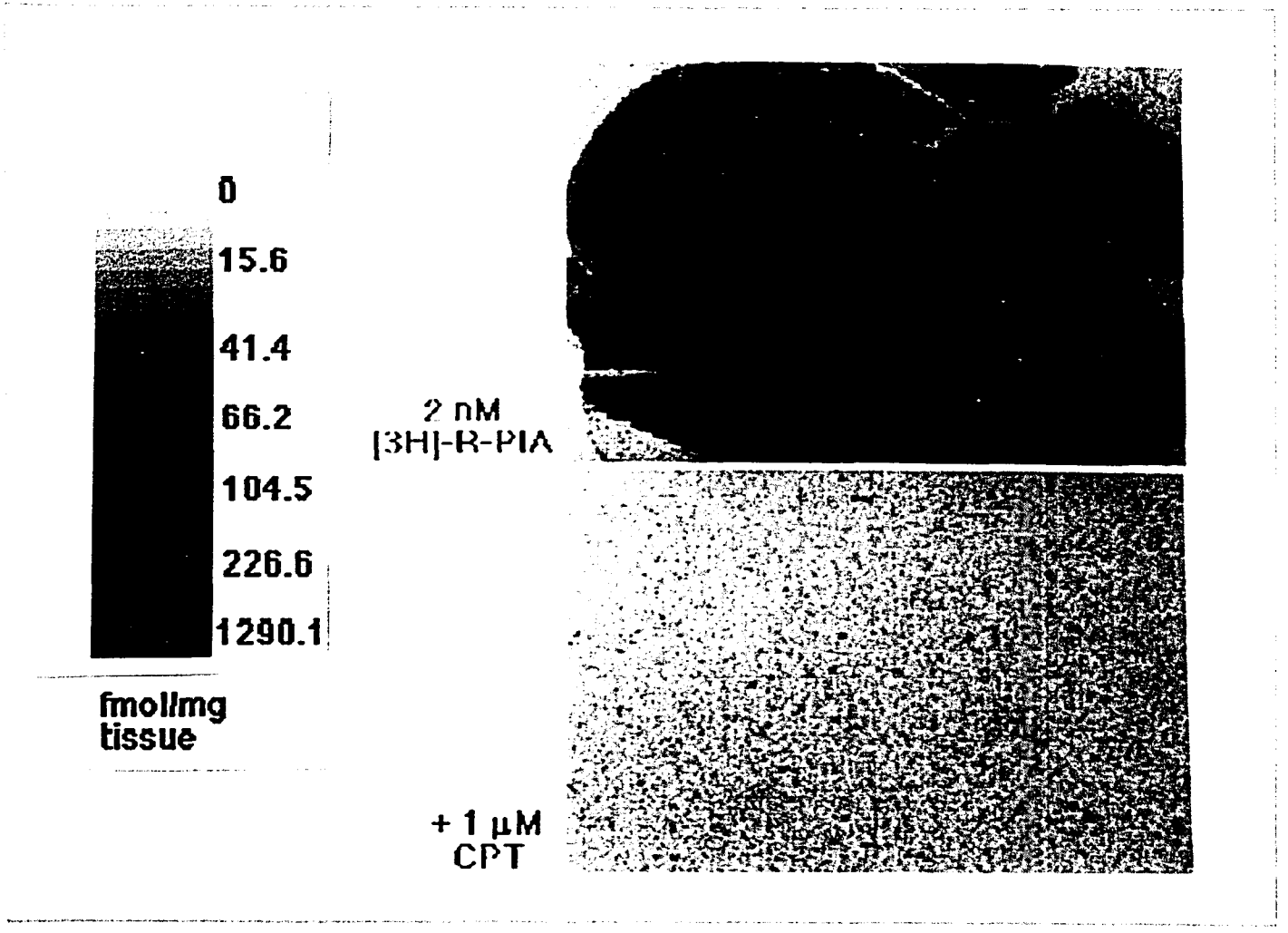

0
15.6
41.4
66.2
104.5
226.6
1290.1
fmol/mg
tissue
2 nM
[3H]-R-PIA
+ 1 µM
CPT

Fig. 3. Illustration of images produced on tritium-sensitive film by [^{3}H]-R-PIA (2 nM) bound to a coronal section of rat brain at the level of the dorsal hippocampus in the absence and the presence of 10 μM CPT Sections (15 μ) were prepared for quantitative receptor autoradiography as described in Materials and Methods, and the sections exposed to the film for 30 days. Binding of [^{3}H]-R-PIA in the presence of CPT ("non-specific binding") was not significantly different from the film background. Binding at the level of CA1 stratum radiatum (300 fmol/mg tissue) and molecular layer of the dentate gyrus (195 fmol/mg tissue) were proportional to the B_{max} values obtained in these regions (Table 3).

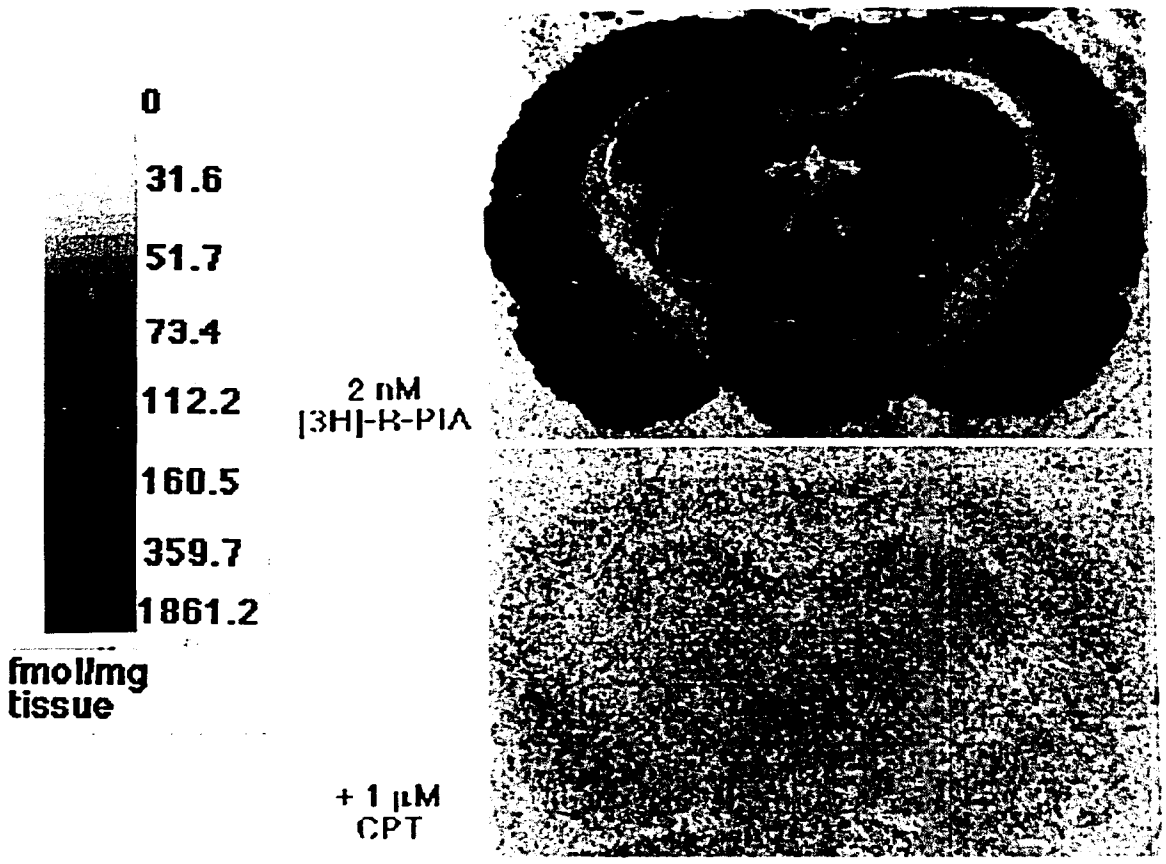

0
31.6
51.7
73.4
112.2
160.5
359.7
1861.2
fmol/mg tissue
2 nM [3H]-R-PIA
+ 1 µM CPT

Fig. 4. Representative images made of (±)-[^{3}H]-8-OH-DPAT bound to a section of human hippocampus. Sections (15 μ) of 6 hr. post-mortem human hippocampus (subject #2, Table 1) were prepared for quantitative autoradiography as described in Materials and Methods. Binding of (±)-[^{3}H]-8-OH-DPAT (2 nM) in the absence and the presence of 1 μM BMY 7378 was assayed, and the sections were exposed to film for 29 days. Binding in the presence of BMY-7378 ("non-specific") was not significantly different from the film background. Binding at the level of CA1 stratum pyramidale (216 fmol/mg tissue), and dentate gyrus (69 pmol/mg tissue) were similar in another 5 samples, and were in proportion to the B_{max} (Table 4).

0
12.9
31.7
82.4
331.1
fmol/mg
tissue
2 nM
[3H]-8-OH-DPAT
+ 1μM
BMY-7378

Fig. 5. Representative images made from (±)-[^{3}H]-8-OH-DPAT bound to a coronal section of rat brain at the level of the dorsal hippocampus. Sections (15 μ) of rat brain were prepared for quantitative receptor autoradiography as described in Materials and Methods. Binding of (±)-[^{3}H]-8-OH-DPAT (2 nM) in the absence and the presence of 1 μM BMY 7378 was assayed, and sections were exposed to film for 25 days. Binding in the presence of BMY-7378 ("non-specific") binding was not significantly different from film background. Binding at the level of CA1 stratum radiatum (159 fmol/mg tissue), and dentate gyrus (192 fmol/mg tissue) were similar in another 3 animals, and were in proportion to the B_{max} values in these regions (Table 5).

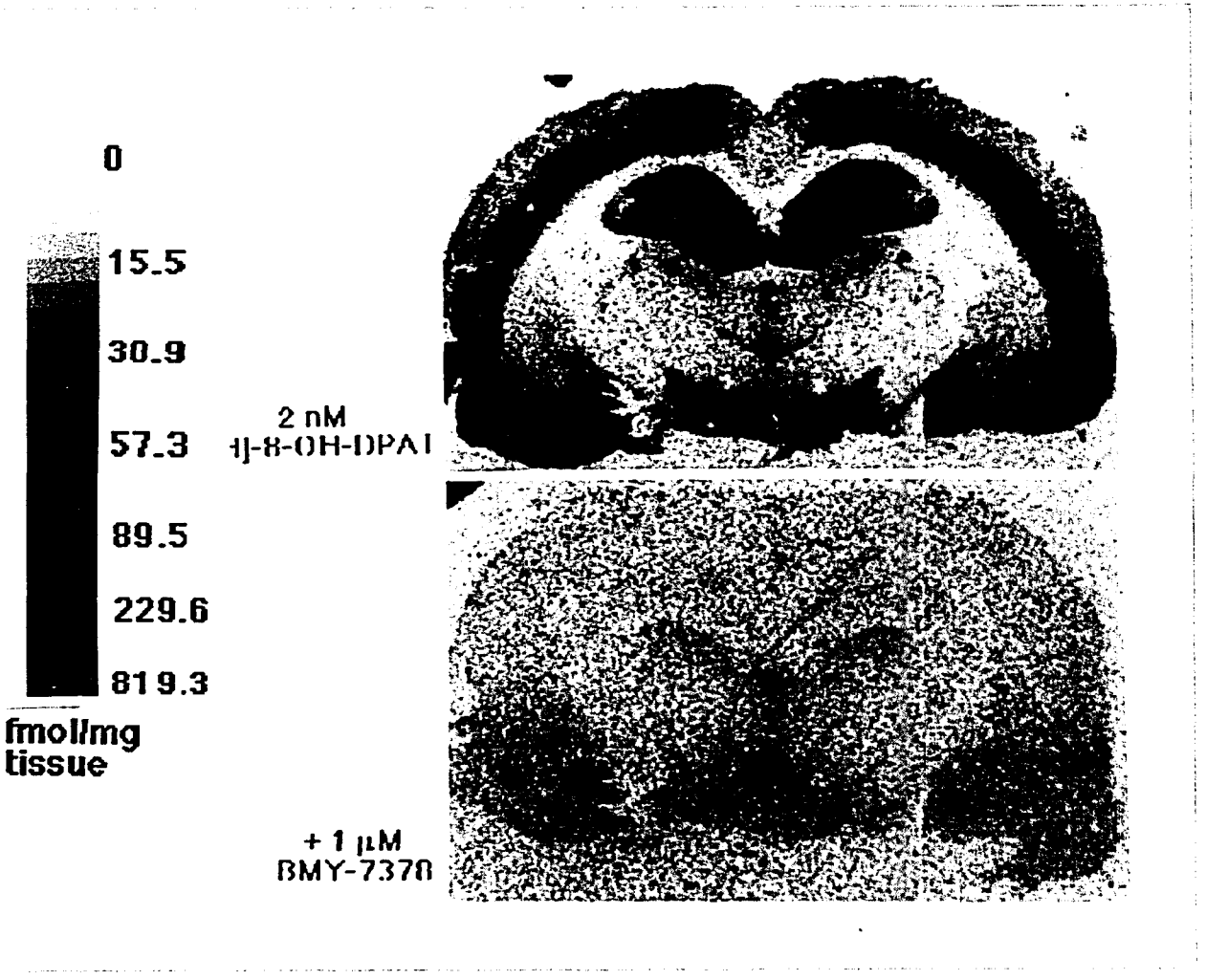
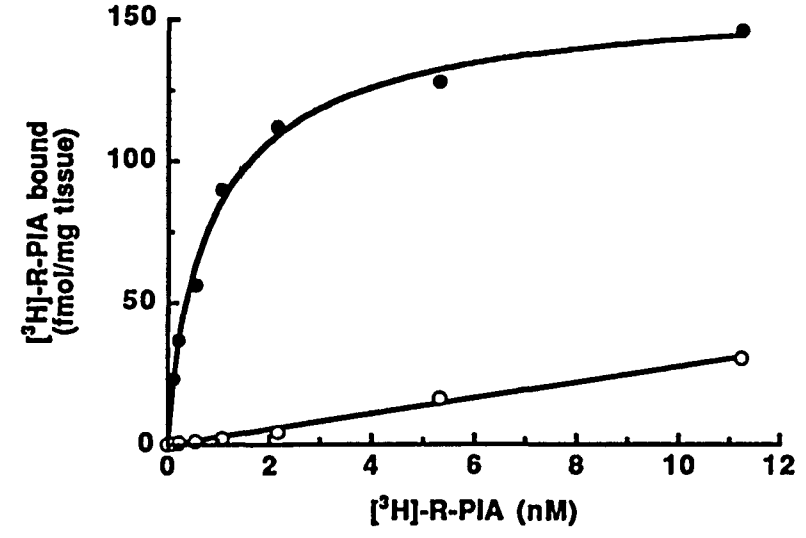

0
15.5
30.9
57.3
89.5
229.6
819.3
fmol/mg
tissue
2 nM
[]-8-OH-DPAT
+ 1 µM
BMY-7378

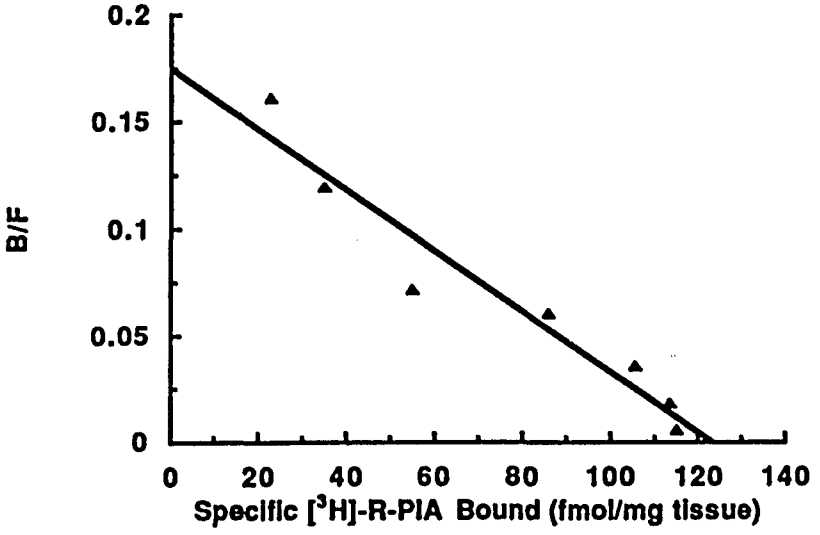
Fig. 6. Representative total and "non-specific" binding, and Rosenthal transformations, of the "specific" binding of [^{3}H]-R-PIA (A,B), (±)-[^{3}H]-8-OH-DPAT (C,D), to rat (B,D) and human (A,C) hippocampal sections, in the sub-region dentate gyrus. Each data point represents the mean of quadruplicate determinations in 1 to 2 tissue slices, with standard deviations less than 10 % of the mean. Estimates of three binding parameters, K_d, B_{max} and Hill coefficient, were obtained from non-linear regression analysis as described in Materials and Methods, and listed in the table below. These experiments were replicated an additional four times in human and an additional one to four times in rat, all of which yielded similar results. A summary of results collected from all experiments done is presented in Tables 2-5

	[^{3}H]-R-PIA		(±)-[^{3}H]-8-OH-DPAT	
	Human	Rat	Human	Rat
K_d (nM)	0.51	0.68	0.41	0.30
B_{max}				
(fmol/mg tissue)	122	201	97	190
(pmol/mg protein)*	1.22	2.01	0.97	1.9
n_H	0.92	0.83	0.91	0.94

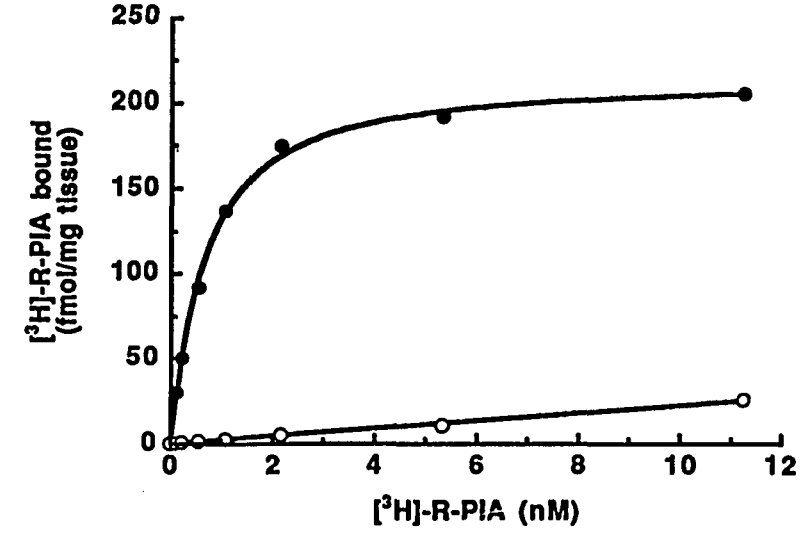
*Values converted from fmol/mg tissue based upon 10% particulate protein(METHODS).

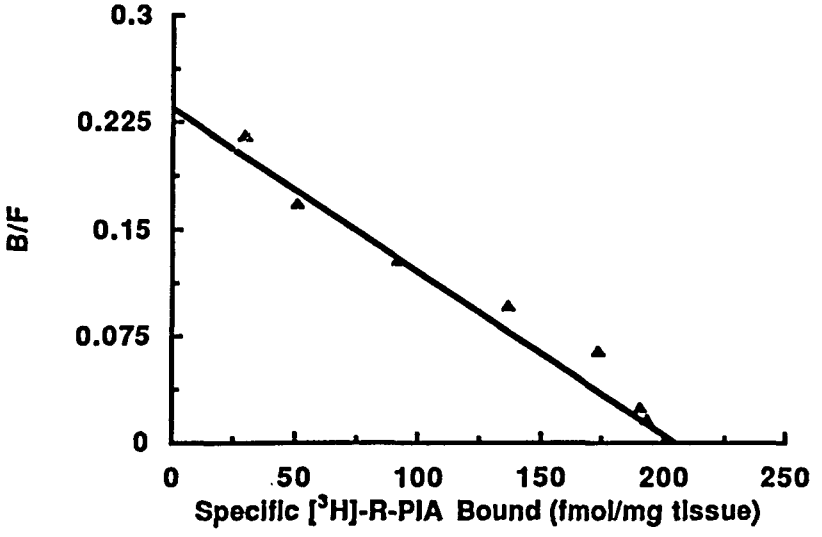
A. [^{3}H]-R-PIA
Human Dentate Gyrus



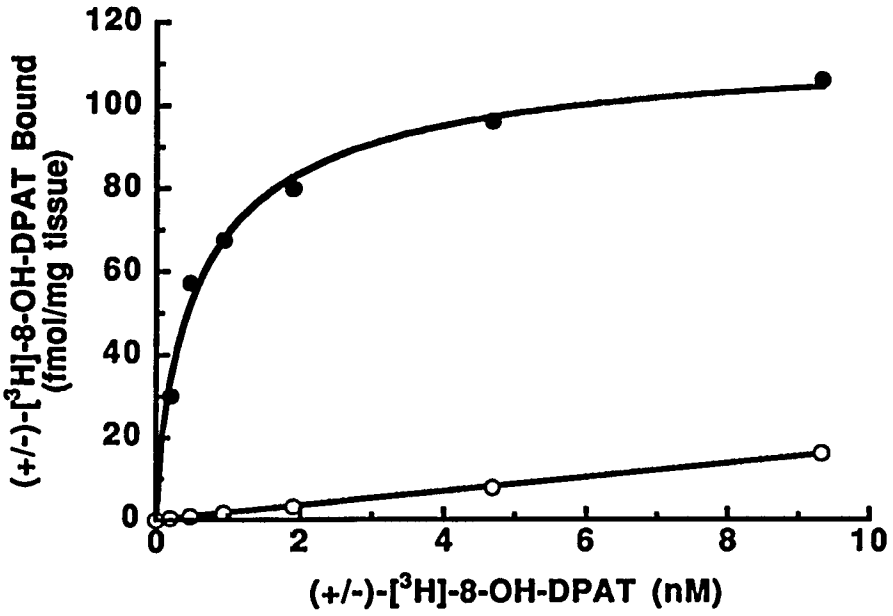


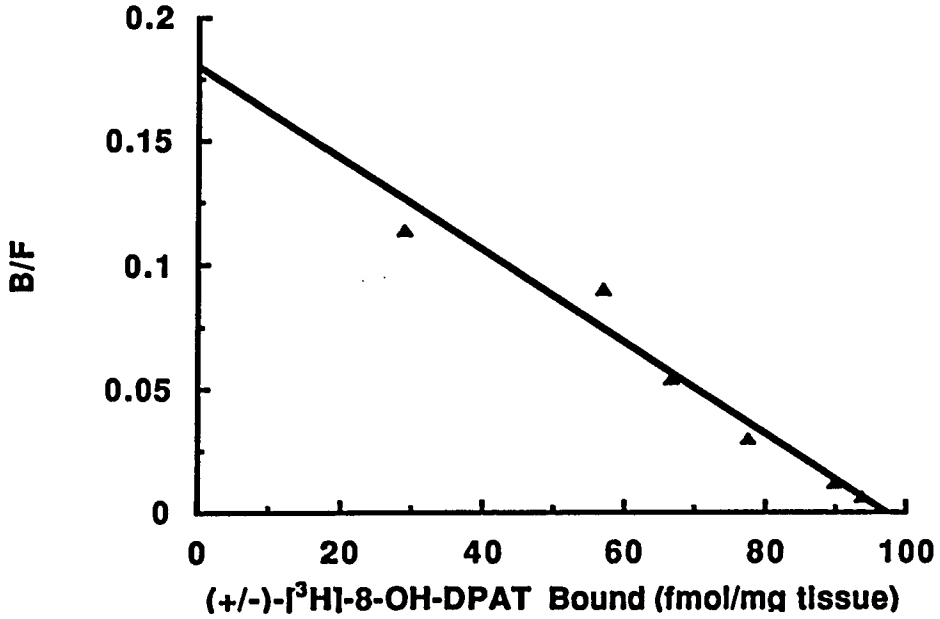
B. [^{3}H]-R-PIA
Rat Dentate Gyrus



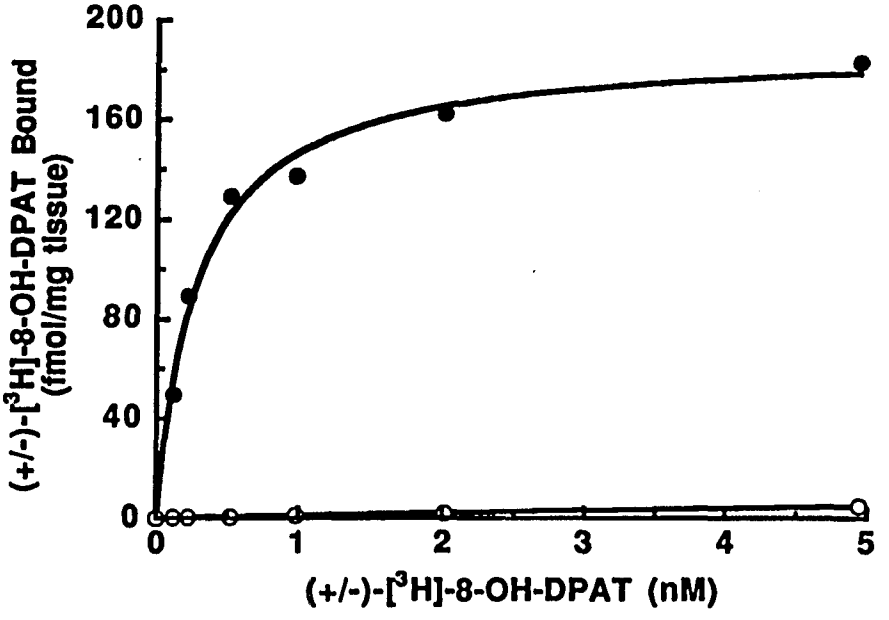


C. (+/-)-[^{3}H]-8-OH-DPAT
Human Dentate Gyrus





D. (+/-)-[^{3}H]-8-OH-DPAT
Rat Dentate Gyrus



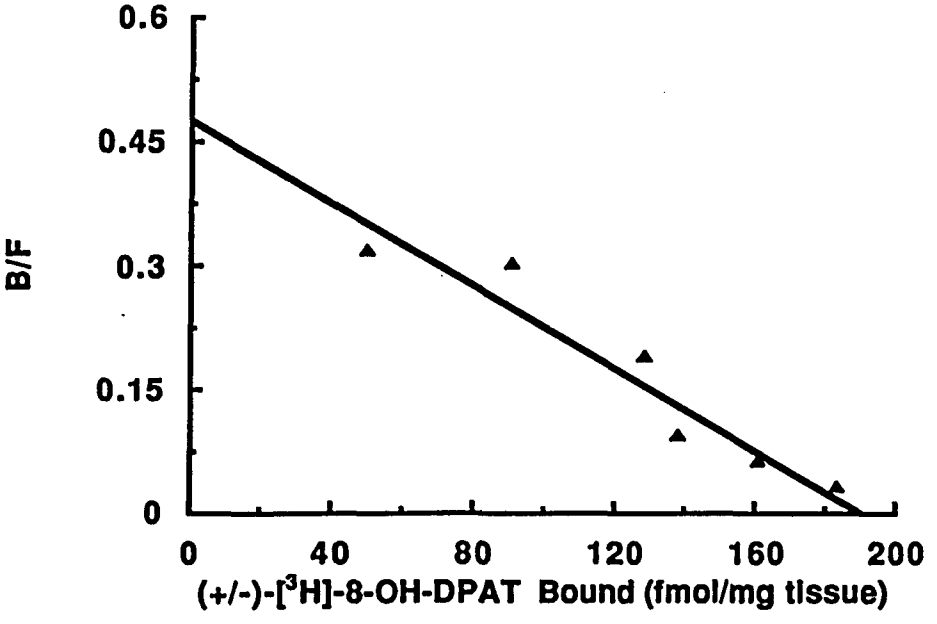


Table 2

[^{3}H]-R-PIA Binding Parameters in Human Hippocampus

Tissue was obtained and processed for quantitative autoradiography as described in Material and Methods. [^{3}H]-R-PIA concentrations were varied from 0.1 nM-10 nM, with non-specific binding defined by 10 μM CPT. Data shown below represent the mean ± SEM for 5 different human samples. In the column illustrating affinity constants, values in parentheses represent arithmetic mean of the K_d values. In the column illustrating receptor density, values in parentheses represent (pmol/mg protein, METHODS).

Region	pK_d *	B_{max} ** (fmol/mg tissue)	n_H***
CA1			
Stratum oriens	9.06±0.20 (0.95 nM)	137±15 (1.37)	0.94±0.11
Stratum pyramidale	8.96±0.18 (1.17 nM)	240±24 (2.4)	0.98±0.05
Stratum radiatum	8.98±0.16 (1.12 nM)	200±24 (2.0)	1.01±0.09
Stratum lacunosum-moleculare	8.81±0.24 (1.74 nM)	109±14 (1.09)	0.81±0.12
CA3			
Stratum oriens	8.96±0.25 (1.25 nM)	143±22 (1.43)	0.95±0.06
Stratum pyramidale	8.93±0.25 (1.33 nM)	112±31 (1.12)	0.90±0.04
Stratum radiatum	8.93±0.22 (1.31 nM)	7.30±1.64	1.05±0.11
Stratum lacunosum-moleculare	9.15±0.38 (0.88 nM)	91.5±35.4 (0.92)	1.04±0.16

Table 2, continued

[^{3}H]-R-PIA Binding Parameters in Human Hippocampus (cont.)

Region	pK_d *	B_{max} ** (fmol/mg tissue)	n_H***
Ca4	8.95±0.28 (1.32 nM)	131±21 (1.31)	0.96±0.06
Dentate gyrus	8.95±0.27 (1.30 nM)	159±21 (1.59)	0.94±0.05
Cortex	8.90±0.24 (1.43 nM)	168±12 (1.68)	0.93±0.04

*No statistical difference in the K_d values of [^{3}H]-R-PIA between the sub-regions of human hippocampus (F=0.51, p=0.87, ANOVA).

**Statistical differences exist in the B_{max} values between subregions of the hippocampus (F=3.93, p≤0.001, ANOVA).

***Hill coefficients are not statistically different from unity (F=0.61, p=0.81, ANOVA).

Table 3

[^{3}H]-R-PIA Binding Parameters in Rat Hippocampus

Data shown below represent the mean ± SD of [^{3}H]-R-PIA saturation analysis (0.1-10.0 nM) in two different animals. Non-specific binding was defined using 10 µM CPT. In the column illustrating affinity constants, values in parentheses represent arithmetic mean of the K_d values. In the column illustrating receptor density, values in parentheses represent (pmol/mg protein, METHODS).

Region	pK_d *	B_{max} ** (fmol/mg tissue)	n_H***
CA1			
Stratum oriens	9.19 (0.65 nM)	254±17 (2.54)	0.84±0.04
Stratum pyramidale	9.04 (0.91 nM)	161±6.3 (1.61)	0.91±0.02
Stratum radiatum	9.08 (0.83 nM)	363±17 (3.63)	0.82±0.06
Stratum lacunosum-moleculare	9.13±0.08 (0.74 nM)	174±1 (1.74)	0.83±0.11
Dentate Gyrus			
Inner blade Molecular	9.08 (0.84 nM)	244±11 (2.44)	0.80±0.09
Outer Blade Molecular	9.12 (0.76 nM)	230±21 (2.3)	0.83±0
Inner blade Granular	9.22 (0.61 nM)	83±8 (0.83)	0.85±0.01
Outer Blade Granular	9.12 (0.77 nM)	84±19 (0.84)	0.90±0.09
Hilus	9.13 (0.75 nM)	175±21 (1.75)	0.84±0.01

Table 3, continued

[^{3}H]-R-PIA Binding Parameters in Rat Hippocampus (cont.)

Region	pK_d	B_{max} (fmol/mg tissue)	n_H
CA3 Stratum oriens	9.24 (0.58 nM)	178±9 (1.78)	0.89±0.13
Stratum pyramidale	9.30 (0.55 nM)	107±28 (1.07)	0.80±0.06
Stratum radiatum	9.20 (0.69 nM)	245±25 (2.45)	0.86±0.18
Medial thalamic nuclei	9.03 (0.95 nM)	220±51 (2.2)	0.83±0.12
Dorsal thalamic nuclei	9.08 (0.82 nM)	143±36 (1.43)	0.84±0.21
Ventral Posterior Thalamic Nuclei	9.10 (0.80 nM)	170±13 (1.7)	0.85±0.13
Cortex	9.17 (0.69 nM)	137±9 (1.37)	0.84±0.04

*No statistical difference in the K_d values of [^{3}H]-R-PIA between the sub-regions of rat hippocampus, or the other brain regions listed (F=0.89, ANOVA).

**Statistical differences exist in the B_{max} values between subregions of the hippocampus and other brain regions (F=9.58, $p \leq 0.001$).

***Hill coefficients are not statisically different from unity (F=0.58, p=0.86, ANOVA).

Table 4

(±)-[^{3}H]-8-OH-DPAT Binding Parameters in Human Hippocampus

Tissue was obtained and processed for quantitative receptor autoradiography as described in Materials and Methods. (±)-[^{3}H]-8-OH-DPAT concentrations were varied from 0.1-10 nM, with non-specific binding defined by 1 μM BMY-7378. Data shown below represent mean ± SEM for 6 different human samples. In the column illustrating affinity constants, values in parentheses represent arithmetic mean of the K_d values. In the column illustrating receptor density, values in parentheses represent (pmol/mg protein, METHODS).

Region	pK_d *	B_{max} ** (fmol/mg tissue)	n_H
CA1 Stratum oriens	9.38 (0.43 nM)	169±13 (1.69)	0.76±0.04
Stratum pyramidale	9.41 (0.40 nM)	278±17 (2.78)	0.80±0.03
Stratum radiatum	9.29 (0.54 nM)	220±22 (2.22)	0.80±0.04
Stratum lacunosum-moleculare	9.30 (0.56 nM)	214±17 (2.14)	0.88±0.06
Ca4	9.38 (0.43 nM)	50±6 (0.5)	0.66±0.03
Dentate	9.44 (0.39 nM)	86±9 (0.86)	0.77±0.03
Inner Cortex	9.41 (0.39 nM)	52±6 (0.52)	0.78±0.09
Outer Cortex	9.41 (0.39 nM)	164±15 (1.64)	0.76±0.03

*No statistical difference in the K_d values of (±)-[^{3}H]-8-OH-DPAT between the sub-regions of human hippocampus (F=0.95, p=0.48, ANOVA).

**Statistical differences exist in the B_{max} values between subregions of the hippocampus (F=33.41, p≤0.001, ANOVA)..

***Hill coefficients statistically different from unity (F=4.46, p≤0.001)

Table 5

(±)-[^{3}H]-8-OH-DPAT Binding Parameters in Rat Hippocampus

Data shown below represent mean ± SEM of (±)-[^{3}H]-8-OH-DPAT saturation analysis (0.1-5 nM, 4 different animals). Non-specific binding was defined using 1 µM BMY-7378. In the column illustrating affinity constants, values in parentheses represent arithmetic mean of the K_d values. In the column illustrating receptor density, values in parentheses represent (pmol/mg protein, METHODS).

Region	pK_d *	B_{max} ** (fmol/mg tissue)	n_H
CA1			
Stratum oriens	9.58 (0.27 nM)	104±2 (1.04)	0.99±0.03
Stratum radiatum	9.53 (0.30 nM)	168±10 (1.68)	0.92±0.04
Stratum lacunosum moleculare	9.50 (0.32 nM)	138±14 (1.38)	0.90±0.05
CA2			
Stratum oriens	9.27 (0.56 nM)	22±2 (0.22)	0.99±0.04
Stratum pyramidale	9.30 (0.60 nM)	18±3 (0.18)	0.95±0.07
CA3			
Stratum oriens	9.40 (0.42 nM)	89±11 (0.89)	0.94±0.09
Stratum pyramidale	9.53 (0.31 nM)	63±6 (0.63)	0.88±0.09

Table 5, continued

(±)-[^{3}H]-8-OH-DPAT Binding Parameters in Rat Hippocampus (cont.)

Region	pK_d *	B_{max} ** (fmol/mg tissue)	n_H
Dentate Gyrus Inner blade	9.50 (0.32 nM)	5.69±0.31	0.94±0.03
Outer Blade	9.54 (0.30 nM)	191±8 (1.91)	0.97±0.05
Hilus	9.54 (0.29 nM)	107±1 (1.07)	0.98±0.08
Cortex	9.53 (0.30 nM)	42±5 (0.42)	0.90±0.07

*No statistical difference in the K_d values of (±)-[^{3}H]-8-OH-DPAT between the sub-regions of rat hippocampus, or the other brain regions listed ($F=1.49$, $p=0.19$, ANOVA).

**Statistical differences exist in the B_{max} values between subregions of the hippocampus and other brain regions ($F=58.17$, $p\leq0.0001$).

***Hill coefficients are not statisically different from unity ($F=0.46$, $p=0.92$, ANOVA).

Guanine Nucleotides Attenuate Agonist Binding to Adenosine A_1 and 5-Hydroxytryptamine$_{1A}$ Receptors: A Quantitative Autoradiographic Study in Rat and Human Hippocampus

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Running Title: Guanine Nucleotide Attenuation of Agonist Binding in Rat and Human Hippocampus

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ABSTRACT

Interaction between hormone receptor and guanine nucleotide binding protein (G-protein) is a required step for the initiation of many signal transduction pathways in excitable cells. A result of this interaction is that binding of the hormone and the guanine nucleotides (GN) to their respective protein is mutually modulated (allosteric interaction). In this study we have characterized the allosteric modulation *in situ* by applying quantitative receptor autoradiography (QRA) in human and rat hippocampus. Two G_i-linked receptors, the adenosine A_1 (AD A_1) and 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) were radiolabeled by the selective agonists (±)-[^{3}H]-8-OH-DPAT and [^{3}H]-R-PIA respectively, in sections from human hippocampus and in coronal sections of rat brain at the level of dorsal hippocampus. Affinity values of (±)-[^{3}H]-8-OH-DPAT to BMY-7378-sensitive sites were similar across the hippocampal subregions of rat and human: (K_d 0.5-1.0 nM). Similarly, affinity values of of [^{3}H]-R-PIA to CPT-sensitive sites were similar across the rat hippocampal subregions (0.6-0.8 nM), but slightly higher in human hippocampal subregions (1.0-1.8 nM). Gpp(NH)p attenuated the binding of these ligands in a concentration and saturable manner. Parameters of the concentration-effect curves (E_{max}, EC_{50}, and slope index) were determined by QRA in both rat and human brain hippocampal sections with a single concentration of each [^{3}H]-agonist (70-80% occupancy). In contrast to the consistent partial attenuation of [^{3}H]-agonist binding produced by saturating Gpp(NH)p concentrations in membrane preparations (50%), in brain sections

from the two species (8-13 regions), we observed a near complete effect. Within regions, the EC_{50} values of the GN curves were indistinguishable for a given drug in a given species, but for (±)-[^{3}H]-8-OH-DPAT this parameter was invariably lower (0.1-0.2 μM) in human than in rat brain sections (0.3-0.4 μM) . Also, in human hippocampus the EC_{50} of the GN curves were lower for (±)-[^{3}H]-8-OH-DPAT than those of [^{3}H]-R-PIA (0.2-0.3 μM). The slope index of the curves did not differ from unity in any region, receptor, or species for both receptors.

An intriguing "asymmetry" in the kinetics of the allosteric interaction between binding of Gpp(NH)p and binding of the [^{3}H]-agonist was observed in all subregions from the two species tested. Addition of Gpp(NH)p to sections when the [^{3}H]-agonist had reached equilibrium, caused a biphasic decrease of the [^{3}H]-agonist binding: a rapid 50% decrease within one minute, followed by additional 20-30% decrease over the the next hour. In contrast, a co-incubation of the brain section with [^{3}H]-agonist in the presence of Gpp(NH)p caused a near complete attenuation of [^{3}H]-agonist binding.

We propose that allosteric interactions between binding of hormone and guanine nucleotide can be characterized by parameters of the Gpp(NH)p concentration-attenuation curve. We have demonstrated in this study that these parameters can be measured reliably *in situ* by applying QRA. This approach enables a comparative anatomical study of different receptor systems across regions and species.

INTRODUCTION

Many membrane bound receptors are members of the large class of receptors which activate a G-protein(s), which in turn cause activation of an effector(s). The most widely studied of this class is the G_s-linked beta-adrenergic receptor (BAR), which stimulates the enzyme effector, adenylyl cyclase. DeLean et al. have proposed for the BAR, a ternary complex model in which the preformed receptor/G-protein complex (R/G) is stabilized by the binding of agonist or hormone (H), through the formation of a high affinity ternary complex (H-R-G) (De Lean et al., 1980). Addition of a guanine nucleotide (GN) which binds to the α subunit of the G-protein, causes destabilization of H-R-G leaving the α–GN subunit free to interact with the effector. The destabilized H-R complex then exhibits a low affinity for the hormone. Thus, binding of GN to the G-protein allosterically affects agonist binding to the receptor, through the interconversion of a high affinity state into a low affinity state of the HRG complex (De Lean et al., 1980). We have previously reported characteristics of this allosterism for two G_i-linked receptors, the adenosine A_1 and the 5-HT_{1A} in a rat hippocampal membrane preparation (CHAPTER 1).

AD A_1 and 5-HT_{1A} receptors are coupled to adenylyl cyclase through inhibitory G-proteins (G_i). Agonist binding to these receptors causes a decrease in the production of cyclic AMP (Ebersolt et al., 1983; Fredholm et al., 1986; De Vivo and Maayani, 1986;

Zgombick et al., 1989). Previously, we have postulated that AD A_1 and 5-HT_{1A} receptors are colocalized on the same hippocampal pyramidal cells and may share a common population of G-proteins (Zgombick, et al., 1989). Agonist binding to both AD A_1 and 5-HT_{1A} receptors has been shown to be modulated by GN (Bruns et al., 1980; Yeung and Green, 1984; Lohse et al., 1984; Gozlan et al., 1983; Schlegel and Peroutka, 1986; and Harrington et al., 1988). Similar to the ternary complex model proposed for the G_s-linked BAR, this modulation may reflect conversion of the high affinity state to the low affinity state.

The initial steps of signal transduction require interaction between at least three proteins embedded in the cell membrane, i.e., the receptor system; the hormone receptor, the G-protein, and an effector. The technique of quantitative receptor autoradiography (Kuhar, et al., 1986) has provided much valuable knowledge of receptor distribution and pharmacology in discrete regions. Studies examining events more distal to the receptor have largely involved the use of membrane preparations. However, autoradiographically, [^{3}H]-Gpp(NH)p has been used to localize G-proteins in rat brain (Gehlert and Wamsley, 1986), adenylate cyclase has been localized using [^{3}H]-forskolin (McMartin and Summers, 1990), and [^{3}H]-phorbol esters used to localize protein kinase C (Zarbin et al., 1989). These techniques has been useful in determining the effect of *in vivo* perturbations such as ischemia (Daval et al., 1989b), chronic drug treatment (Daval et al., 1989a), and pathology (Dewar et al., 1990;

Kerwin and Beats, 1990) on localization and quantitation of individual components of the receptor system.

Destabilization of the H-R-G complex has been visualized autoradiographically, for several receptors, adenosine A_1 (Fastbom and Fredholm, 1990b), adenosine A_2 (Parkinson and Fredholm, 1990), opioid (Zarbin et al., 1990), beta-adrenergic (Zarbin et al., 1983), peptide (Dam and Quirion, 1986; Torda and Saavedra, 1990), and 5-$HT_{1A,1B}$ (Sijbesma et al., 1990). We have quantitated characteristics in the allosterism existing between hormone binding to receptor and guanine nucleotide binding to G-protein, in discrete regions of rat and human hippocampus for two different receptors. These studies have shown that destabilization of H-R-G by guanine nucleotides can be quantitated as a function of region, receptor and species. These studies also illustrate that in human post-mortem tissue, the hormone stabilizes a high affinity ternary complex that is destabilized by guanine nucleotides.

MATERIALS AND METHODS

Tissue Preparation. Male Sprague Dawley rats (200-250 g, Charles River, Boston, MA) were asphyxiated using CO_2 and decapitated. The brain was removed from the skull, immediately frozen on dry ice and stored at -80°C until sectioned. Human brain tissue was obtained from the Department of Neuropathology at Mt. Sinai Medical Center (New York, NY). The history of human brain tissue used in these studies are as described in CHAPTER 2 (Table 1).

Tissue preparation for quantitative receptor autoradiography is as previously described (CHAPTER 2, METHODS). Briefly, brain tissue was sectioned, 15 μ thick, in a cryostat at -20°C. Sections were thaw mounted on cold (-20°C) gelatin coated slides (0.5% gelatin, 0.05% chromium potassium phosphate). Sectioned tissue was dried under vacuum and stored at -20°C until use (generally less than one month). Prior to assay, slides were warmed to room temperature on a heating plate.

High Affinity Binding Assay. The *in vitro* binding of [^{3}H]-R-PIA, and (±)-[^{3}H]-8-OH-DPAT to tissue sections was performed by modification of the method of Pazos et al. (Pazos et al., 1988). Briefly, slides were preincubated at room temperature for 15 min. in assay buffer, (50 mM HEPES, 0.1% ascorbate, 2 mM EGTA, 2.5 mM $MgCl_2$, pH adjusted to 7.4 at room temperature, with saturated Tris

base). For [^{3}H]-R-PIA binding, 2 IU/ml, adenosine deaminase was added, to remove endogenous adenosine (Linden, 1989).

Slide mounted tissue sections (1-2 sections/slide) were incubated at room temperature in 40 ml of [^{3}H]-agonist ([^{3}H]-R-PIA (36-42 Ci/mmol) or (±)-[^{3}H]-8-OH-DPAT (125-142 Ci/mmol)), [^{3}H]-agonist in the presence of Gpp(NH)p (0.1 nM to 100 μM), or the [^{3}H]-agonist plus the respective masking ligand, diluted in assay buffer. Preliminary kinetic studies have demonstrated the association of agonist to receptor to approach equilibrium within 60 mins. (data not shown), therefore, incubations of tissue slices with radioactive drug, were conducted for 60 mins. Non-specific binding was defined by 10 μM CPT ([^{3}H]-R-PIA binding), or 1 μM BMY-7378 ((±)-[^{3}H]-8-OH-DPAT binding). After 60 min. incubation in the respective radioisotope, the slides were washed two times, 5 mins. each, in ice cold assay buffer (4^{o}C), rinsed in ice cold distilled water, and dried under a stream of cold dry air.

For kinetic analysis, slides were preincubated for 15 min. at room temperature in assay buffer, and the association of radioligand followed for 60-120 min. At equilibrium, the 40 mls of radioligand was removed and replaced with 40 mls of radioligand in the presence of either 100 μM Gpp(NH)p or 1 μM BMY-7378 ((±)-[^{3}H]-8-OH-DPAT binding) or 10 μM CPT ([^{3}H]-R-PIA binding). Control values for these experiments were tissue slices which were not preincubated in the respective radioligand, but were co-exposed to [^{3}H]-agonist in the presence of either 100 μM Gpp(NH)p or 1 μM

BMY-7378 or 10 μM CPT. Slides were washed two times, 5 mins. each, in ice cold assay buffer (4°C), rinsed in ice cold distilled water, and dried under a stream of cold dry air.

To determine that the nearly complete attenuation of [^{3}H]-agonist binding by Gpp(NH)p was not artifactual, due to the non-linearity of Hyperfilm, parallel studies were conducted where tissue sections were "wiped" from the slides using filter paper and counted in a scintillation counter. To further rule out this phenomenon, tissue sections were re-exposed for 3 months (normal exposure time 3-4 weeks), in order to increase the optical density of non-specific binding, and binding in the presence of 100 μM Gpp(NH)p. As a third test, slides containing slices that had undergone *in vitro* binding, were broken directly into scintillation vials and counted. In all cases, both slides which had been exposed to [^{3}H]-agonist + masking ligand, or those exposed to [^{3}H]-agonist + a saturating concentration of Gpp(NH)p, displayed very low binding, and were essentially identical.

Quantitative Receptor Autoradiography. Autoradiograms were obtained as described previously (CHAPTER 2). Briefly, dried tissue slices were exposed to tritium sensitive film (HYPERFILM, Amersham, Arlington Heights, IL) at 4° C, for 25 to 45 days. Quantitation of bound radioactivity was determined with tritium standards (AMERSHAM, [^{3}H]-microscales). Films were developed using Kodak D-19 developer, fixed in Kodak Rapid Fixer, rinsed, and allowed to dry at room temperature.

Data Analysis. Optical measurements were transferred to a MacIntosh II computer, as described in CHAPTER 2. Conversion of optical density into density of ligand binding sites was done using IMAGE 1.33 (Wayne Rasband, NIH, Bethesda, MD). Optical density measurements were converted to DPM/mg tissue or fmol/mg tissue, using the [^{3}H]-microscales which contain conversions for tissue equivalents (Geary, et al., 1985. The standards were best fit by a four parameter logistic equation (David Rodbard, NIH, Bethesda, MD and Cary Mariash, Univ. of Minnesota):

$$y = D + \frac{A-D}{1+(X/C)^B}$$

Where, the parameters are represented by:

A = Film Background

B = Slope Index

C = Midpoint of the curve

D = Maximal density

X = The concentration of radioactivity

The final values obtained from the standard curve were then converted from fmol/mg tissue to fmol/mg protein or pmol/mg protein, assuming tissue composition of 10% particulate protein

(CHAPTER 2, METHODS). Non-specific binding was subtracted from total binding to generate specific binding. Identification of the brain regions as well as specific cell layers was made with the aid of cresyl violet stained sections, and identified according to standard neuroanatomical atlases (Duvernoy, 1988; Paxinos and Watson, 1986; DeArmond, et al., 1976; and Barr and Kiernan, 1983).

Guanine nucleotide inhibition of agonist binding was analyzed by fitting a four parameter logistic equation to the data. The equation used was:

$$R = [R_i-(R_i-R_o)]/[([A]/EC_{50})^n+1]$$

where,

R = The amount of [^{3}H]-agonist bound (pmol/mg protein) in the presence of a specified concentration of guanine nucleotide.

R_o = The amount of [^{3}H]-agonist bound (pmol/mg protein) in the absence of guanine nucleotide.

R_i = The amount of [^{3}H]-agonist bound (pmol/mg protein) in the presence of a saturating concentration of guanine nucleotide.

A = The concentration of guanine nucleotide mediating the response.

EC_{50} = The concentration of guanine nucleotide eliciting a half maximal inhibition of the response.

n = The slope index for the concentration response curve.

Statistical evaluation was done using one way ANOVA, followed by Duncan's multiple range test. The accepted level of significance was $p \leq 0.05$.

Materials. (±)-[^{3}H]-8-OH-DPAT (125-140 Ci/mmol) and [^{3}H]-R-PIA (36-42 Ci/mmol), were purchased from New England Nuclear (Boston, MA.) CPT was purchased from Research Biochemicals, Inc. (Natick, MA). BMY-7378 was a generous gift from Dr. Frank Yocca, of Bristol-Myers Squibb Co. (Wallingford, CT). Gpp(NH)p and all other chemicals used in assay buffer and in gelatin coating the slides, were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

RESULTS

Attenuation of [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT binding to rat and human hippocampal sections by Gpp(NH)p. At a saturating concentration, Gpp(NH)p (100 μM) attenuated up to 95% of [^{3}H]-R-PIA (2 nM) binding to sections of human hippocampus (Fig. 1), and coronal sections of rat brain at the level of the dorsal hippocampus (Fig. 2). In both species, the amount of Gpp(NH)p-insensitive [^{3}H]-R-PIA binding was similar to the CPT-insensitive [^{3}H]-R-PIA binding ("non-specific" binding), and both quantities were not significantly different from the film background. A qualitatively similar effect of 100 μM Gpp(NH)p on (±)-[^{3}H]-8-OH-DPAT (2 nM) binding was observed in both species (Figs. 3-4). Identical assay conditions were utilized for both [^{3}H]-agonists, and assays were done in adjacent brain slices. Although qualitatively similar, these results are quantitatively different from those observed in rat hippocampal membrane preparations (CHAPTER 1), where maximal attenuation was approximately 50% of control (see below).

We further characterized the Gpp(NH)p effect by constructing complete concentration-effect curves for the attenuation in binding of [^{3}H]-R-PIA in human hippocampus, and (±)-[^{3}H]-8-OH-DPAT in both rat and human hippocampus. Adjacent brain sections were co-incubated with the [^{3}H]-agonists and Gpp(NH)p concentrations. Based on our previous studies with the two [^{3}H]-agonists, (CHAPTER 2), we chose 2 nM of either drug because it produces approximately 75-80% occupancy of the respective receptor sites. Thirteen concentrations

of Gpp(NH)p (1 nM - 100 μM) were tested with each drug. Representative results for the Gpp(NH)p attenuation of [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT are shown in Fig. 5 (human stratum pyramidale). Fig. 6 illustrates the attenuation of (±)-[^{3}H]-8-OH-DPAT binding in a coronal section of rat dorsal hippocampus, dentate gyrus, as compared to human dentate gyrus.

In both species Gpp(NH)p attenuated the binding of the two agonists in a concentration-dependent and saturable manner. Three parameters of the concentration-effect curves (maximal effect, EC_{50}, and slope index) were evaluated by non-linear regression analysis (Materials and Methods) and are presented in Tables 1-3. Maximal attenuation of [^{3}H]-R-PIA (Table 1) and (±)-[^{3}H]-8-OH-DPAT binding (Table 2) in all subregions of the human hippocampus was 89-96%. Significantly lower maximal inhibition was observed with (±)-[^{3}H]-8-OH-DPAT in most subregions of rat hippocampus: 73-96% (Table 3) The EC_{50} values were very similar across the tested brain regions: 0.14-0.43 μM ([^{3}H]-R-PIA; human), 0.15-0.48 μM ((±)-[^{3}H]-8-OH-DPAT; human) and 0.26-0.45 μM ((±)-[^{3}H]-8-OH-DPAT; rat) Although not statistically different, a slightly greater potency (lower EC_{50} values) of this effect in the 5-HT_{1A} receptor system was consistent in most subregions of the human hippocampus, when compared to [^{3}H]-R-PIA in the same subregion. The [^{3}H]-DPAT attenuation in most subregions of human hippocampus generated EC_{50} values less than the comparable region of rat hippocampus ($p \leq 0.001$, dentate gyrus and stratum lacunosum). The slope index of all Gpp(NH)p curves were not different from unity.

Comparison of the Gpp(NH)p attenuation of (±)-[^{3}H]-8-OH-DPAT binding in rat hippocampus: Membrane homogenates versus brain sections. We have reported previously on the attenuation of (±)-[^{3}H]-8-OH-DPAT binding in rat hippocampal membranes by Gpp(NH)p (CHAPTER 1). A comparison was made between the previous study using membrane preparations and brain sections which were assayed under identical experimental conditions. Because K_d values of (±)-[^{3}H]-8-OH-DPAT binding in membranes (0.4 nM; CHAPTER 1) and brain sections (0.3-0.4 nM in most subregions; CHAPTER 2) are identical, a comparison of the Gpp(NH)p effects at 2 nM (±)-[^{3}H]-8-OH-DPAT (about 80% occupancy) under identical experimental conditions should provide the required ground for a comparative study between the two brain preparations. As shown in Fig. 7, the EC_{50} of Gpp(NH)p effect in a membrane preparation, 0.47 ± 0.04 is comparable to the values obtained using quantitative receptor autoradiography, in all subregions of rat hippocampus (Fig. 7, and Table 3). Two significant differences, however, were consistently observed between the two preparations: a) confirming our previous report (CHAPTER 2), the density of BMY-7378-sensitive (±)-[^{3}H]-8-OH-DPAT sites in most subregions of the hippocampus (Table 3) was much higher (range in subregions 0.2-2.0 pmol/mg protein) than in the membrane preparation (1.04±0.03 pmol/mg protein, n=3); and b) the maximal attenuation attainable was higher in the brain section (Fig. 7, Table 3, and CHAPTER 1). While Gpp(NH)p decreased only half of the (±)-[^{3}H]-8-OH-DPAT binding in rat hippocampal membranes (50 ± 3 %, n=3, CHAPTER 1), the maximal attenuation in

most subregions of the rat hippocampus were approximately at least 75 to 95%. The E_{max} in the membrane preparation was significantly less ($p \leq 0.01$) in hippocampal membrane homogenates, than all measured subregions in the 15 μ section of rat hippocampus.

Kinetics of the Gpp(NH)p effect. The kinetics of the Gpp(NH)p effect were assayed after an equilibrium of [^{3}H]-agonist binding was obtained. At that time, either a masking ligand (1 μM BMY-7378 or 10 μM CPT) or Gpp(NH)p (100 μM) were added. Illustrated in Figs. 8-9 is the kinetic profile obtained with [^{3}H]-R-PIA in a subregion of human hippocampus (stratum pyramidale). An equilibrium of 2 nM [^{3}H]-R-PIA binding was observed within 80 min (200 fmol/mg tissue). A subsequent addition of 10 μM CPT initiated a very slow dissociation: during the first 60 min. 54 fmol/mg tissue of bound [^{3}H]-R-PIA were dissociated. In contrast, the addition of 100 μM Gpp(NH)p to adjacent sections assayed in parallel, elicited a biphasic displacement: a very rapid initial dissociation (the first point, collected at 1 min., revealed a decrease to 108 fmol/mg tissue of [^{3}H]-R-PIA binding), followed by a very slow process: within the next 60 min. an additional 36 fmol/mg tissue were dissociated. In parallel control assays sections which were co-incubated with either [^{3}H]-R-PIA and 100 μM Gpp(NH)p or with [^{3}H]-R-PIA and 10 μM CPT displayed substantially less binding, and were indistinguishable from film background. This apparent time-related "asymmetry" in Gpp(NH)p attenuation of [^{3}H]-R-PIA binding was consistently observed in 15 μ sections of human cerebellum or in coronal sections of rat brain (data not shown).

Similar kinetics were observed with (±)-[^{3}H]-8-OH-DPAT binding. (Figs. 10-11). At equilibrium, [^{3}H]-8-OH-DPAT labeled 291 fmol/mg tissue in the human hippocampus, stratum pyramidale. Upon the subsequent addition of 1 μM BMY-7378, the bound drug was displaced completely within 30 min. The addition of 100 μM Gpp(NH)p elicited a biphasic dissociation: a rapid 57% dissociation occurred within 1 min. (to 126 fmol/mg tissue), followed by a very slow decay over the next 60 min. (final value 69 fmol/mg tissue). In control assays, slides which were co-incubated with (±)-[^{3}H]-8-OH-DPAT and 100 μM Gpp(NH)p or with (±)-[^{3}H]-8-OH-DPAT and 1 μM BMY-7378 displayed very low levels of binding (15 fmol/mg tissue). As with [^{3}H]-R-PIA, this apparent time-related "asymmetry" in Gpp(NH)p attenuation of (±)-[^{3}H]-8-OH-DPAT binding was observed consistently with all brain sections tested (data not shown).

DISCUSSION

The goal of the present study was to identify, in brain sections, characteristics of the allosteric interaction(s) between binding of two ligands, guanine nucleotide and hormone, to a precoupled receptor-G-protein complex. This relationship was studied for two G_i-linked receptors, the 5-HT_{1A} and the AD A_1 in subregions of the hippocampus of two species, human and rat. Two assumptions were made: 1. Similar to membrane bound and solubilized 5-HT_{1A} and AD A_1 receptors, in brain sections at least a portion of these receptors are precoupled to a G-protein(s); and 2. In the absence of guanine nucleotide the hormone forms a ternary complex with the receptor-G-protein complex.

According to the ternary complex model of G-protein-linked receptors, a spontaneous 'pre-coupling' can occur between the G-protein and the receptor in the absence of hormone (De Lean et al., 1980). Indeed, solubilization of the the AD A_1 (Stiles, 1988; Munshi and Linden, 1989) and the 5-HT_{1A} (El Mestikawy et al., 1988; Emerit et al., 1990) receptors has shown both to be associated with G-proteins. We have previously reported that the G_i-linked AD A_1 and 5-HT_{1A} receptors (Zgombick et al., 1989) form a ternary complex with the radiolabeled hormones (H-R-G) in the absence of guanine nucleotides, in a rat hippocampal membrane preparation (CHAPTER 1) and in sections from rat and human brain (Chapter 2). Once formed, the H-R-G ternary complex is rapidly destabilized by guanine

nucleotides, such as Gpp(NH)p, with $t_{1/2}$ less than 10 seconds in both membranes (unpublished data) and in brain sections (Fig.9-10).

We have reported on the high density of both 5-HT_{1A} and AD A_1 receptors in rat hippocampal membranes (CHAPTER 1), where they mediate a concentration-dependent inhibition of adenylyl cyclase activity (Zgombick et al., 1989) Because these receptors are located in the same region and since they activate at least two common effectors (inhibition of adenylyl cyclase activity and opening of K^+ channels) in a non-additive manner, we have previously postulated that they may share components of the signal transduction pathway (Zgombick et al., 1989). In this report we extended our previous studies using quantitative receptor autoradiography. The major advantage in this type of study of is that it provides discrete localization of the destabilization of the ternary complex by Gpp(NH)p for both receptors in adjacent sections of brain tissue

Effects of Guanine Nucleotides on Receptor / G-protein interaction. In membrane preparations, guanine nucleotides have been reported to alter agonist binding to the AD A_1 and 5-HT_{1A} receptors by either decreasing affinity (Schlegel and Peroutka, 1986;, Stiles, 1988), decreasing density of binding sites (Herrick-Davis and Titeler, 1988), or decreasing both affinity and density (Yeung and Green, 1983; Harrington and Peroutka, 1990). Similar studies on the effect of guanine nucleotides on [^{3}H]-5-HT (Sijbesma et al., 1990) or [^{3}H]-cyclohexyladenosine binding (Fastbom and Fredholm, 1987;

Fastbom and Fredholm, 1990b) in coronal sections from rat brain were reported. However, these studies did not fully characterize the concentration dependence of the allosterism. In this study, the characteristics of the allosterism were evaluated with regard to receptor, species, and subregion of the hippocampus.

In a rat hippocampal membrane preparation, we have previously fully characterized the allosterism occurring between hormone binding to receptor and Gpp(NH)p binding to the G-protein(s). This characterization involved attenuation in [^{3}H]-agonist binding in the presence of Gpp(NH)p. In a membrane preparation we could not completely attenuate agonist binding to the receptor-G-protein complex (CHAPTER 1). In contrast, in the sections from rat and post-mortem human hippocampus, we report here the near complete attenuation of agonist binding by 10-100 μM Gpp(NH)p (Fig.1-4, Tables 1-3). Because the ratio of G-protein/receptor in brain tissue has been shown to be quite large (Neubig, et al., 1988), and since all receptor sites appear to be homogeneous with respect to agonist binding (CHAPTER 2), the entire H-R-G population should be equally affected by Gpp(NH)p. It could be expected that Gpp(NH)p would completely destabilize all H-R-G formed. It is not clear why this property was preserved in 15 μ sections of brain tissue, at times up to 12 hr. post mortem, yet not in membrane preparations prepared from fresh rat hippocampus.. The actual preparation of the membrane homogenate by polytronization may introduce artifact into the receptor / G-protein interaction. A substantial decrease of the B_{max} values of dopamine and muscarinic receptor sites by

homogenization with a polytron has been reported (Norman, et al., 1989). Thus, one possibility is that the brain section preserves an interaction between receptor/G-protein coupling that homogenization destroys. This is evidenced in the greater density of binding sites in hippocampal section (CHAPTER 2, and Fig. 7.)

Allosteric Interactions Between Hormone Receptors and Guanine Nucleotides. We assume that the hormone receptor sites and guanine nucleotide G-protein sites are distinct sites on different proteins. Therefore, the observed attenuation of both [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT binding in brain sections reported herein, is proposed to be an allosteric interaction. We have made an extensive characterization of the allosterism between hormone binding to receptor and guanine nucleotide binding to G-proteins, by measuring their mutual effects at different ligand concentrations in membrane preparations from rat hippocampus (CHAPTER 1). Similar to the membrane preparation, binding of the two agonists were attenuated by Gpp(NH)p, in a concentration-dependent and saturable manner (Fig. 5).

The slightly greater sensitivity of [^{3}H]-R-PIA to guanine nucleotides than that of (±)-[^{3}H]-8-OH-DPAT in human hippocampus, (Fig. 1,3) may indicate intriguing differences occurring between the ternary complexes formed with the two receptors. For example, while both receptors may share a common pool of G-proteins (Zgombick et al., 1989), the AD A_1 system may have access to a greater number or other types of G-proteins.

The largest difference in sensitivity to guanine nucleotides was exhibited between human and rat hippocampus. This was seen in two different characteristics; the maximal attenuation of (±)-[^{3}H]-8-OH-DPAT binding was greater in human (a range of 88-90% with region) than rat hippocampus (approximately 75-80% with region) and region for region, the EC_{50} values were lower in human than rat hippocampus. The greater attenuation of (±)-[^{3}H]-DPAT binding in human tissue cannot be due to the post-mortem delay, as Gpp(NH)p attenuated [^{3}H]-R-PIA binding in both human and rat hippocampus, essentially to the level of non-specific binding This raises possibilities that the receptor or G-protein is somehow variable between species, or that their interactions are species related. The greater potency in the (±)-[^{3}H]-8-OH-DPAT attenuation in human hippocampus, compared to the rat hippocampus, indicates a greater affinity of Gpp(NH)p for the H-R-G complex in the human. One might infer again that the receptor or the G-protein or their interaction differs between species.

Kinetic studies of the destabilization of H-R-G by Gpp(NH)p examined autoradiographically. We have previously observed in rat hippocampal membranes a very rapid attenuation of bound (±)-[^{3}H]-8-OH-DPAT or [^{3}H]-R-PIA by Gpp(NH)p (unpublished data). In this preparation, a substantial portion of [^{3}H]-agonist binding, about 50% of either drug was Gpp(NH)p insensitive (CHAPTER 1). As shown in Figs. 1-4, and summarized in tables 1-3, virtually all [^{3}H]-agonist was attenuated by Gpp(NH)p in both species. Therefore, quantitative

autoradiography appears to be a system of choice for kinetic investigation of the allosterism between Gpp(NH)p and drug binding. An intriguing asymmetry in the Gpp(NH)p effect on binding of both [^{3}H]-agonists occurred in both species (Figs. 8-11). This may indicate that the binding of (±)-[^{3}H]-8-OH-DPAT and [^{3}H]-R-PIA to their receptor creates at least two populations of hormone/receptor/G-protein complex; a population that can be rapidly destabilized and a slowly affected population. This heterogeneity can only be observed if the ternary complex is formed first, before being destabilized with Gpp(NH)p.

In summary, we have reported on the allosteric interactions between an agonist and guanine nucleotide, for two different receptors and two different species, examined autoradiographically. For a given drug in a given brain region, uniform characteristics of Gpp(NH)p concentration-effect curves (EC_{50} and E_{max}) were observed. However, in human brain, bound [^{3}H]-R-PIA was more sensitive to Gpp(NH)p than bound (±)-[^{3}H]-8-OH-DPAT, and (±)-[^{3}H]-8-OH-DPAT was more sensitive in human than rat hippocampus. However, for both receptors, destabilization of H-R-G was more complete in the autoradiographic preparation, that in membrane homogenates. We propose that quantitative receptor autoradiography is a valuable technique to investigate characteristics of allosteric interactions between a hormone receptor and G-protein. Furthermore, the integrity of this system is preserved in post-mortem human brain tissue.

Fig. 1. Attenuation of [^{3}H]-R-PIA binding to sections of human hippocampus by Gpp(NH)p. Conditions of the assay are as described in Materials and Methods. Top Panel: [^{3}H]-R-PIA (2 nM) labeled 135 fmol/mg tissue in CA1 stratum pyramidale and 79 fmol/mg tissue in the dentate gyrus. Bottom left panel: Co-incubation of an adjacent hippocampal section with [^{3}H]-R-PIA and 100 μM Gpp(NH)p resulted in binding of 10 and 5 fmol/mg tissue, in the pyramidal layer and dentate gyrus, respectively. Bottom right panel: Co-incubation of a third adjacent section with [^{3}H]-R-PIA and 10 μM CPT resulted in a background level of binding. This experiment was repeated in four additional human samples yielding similar results.

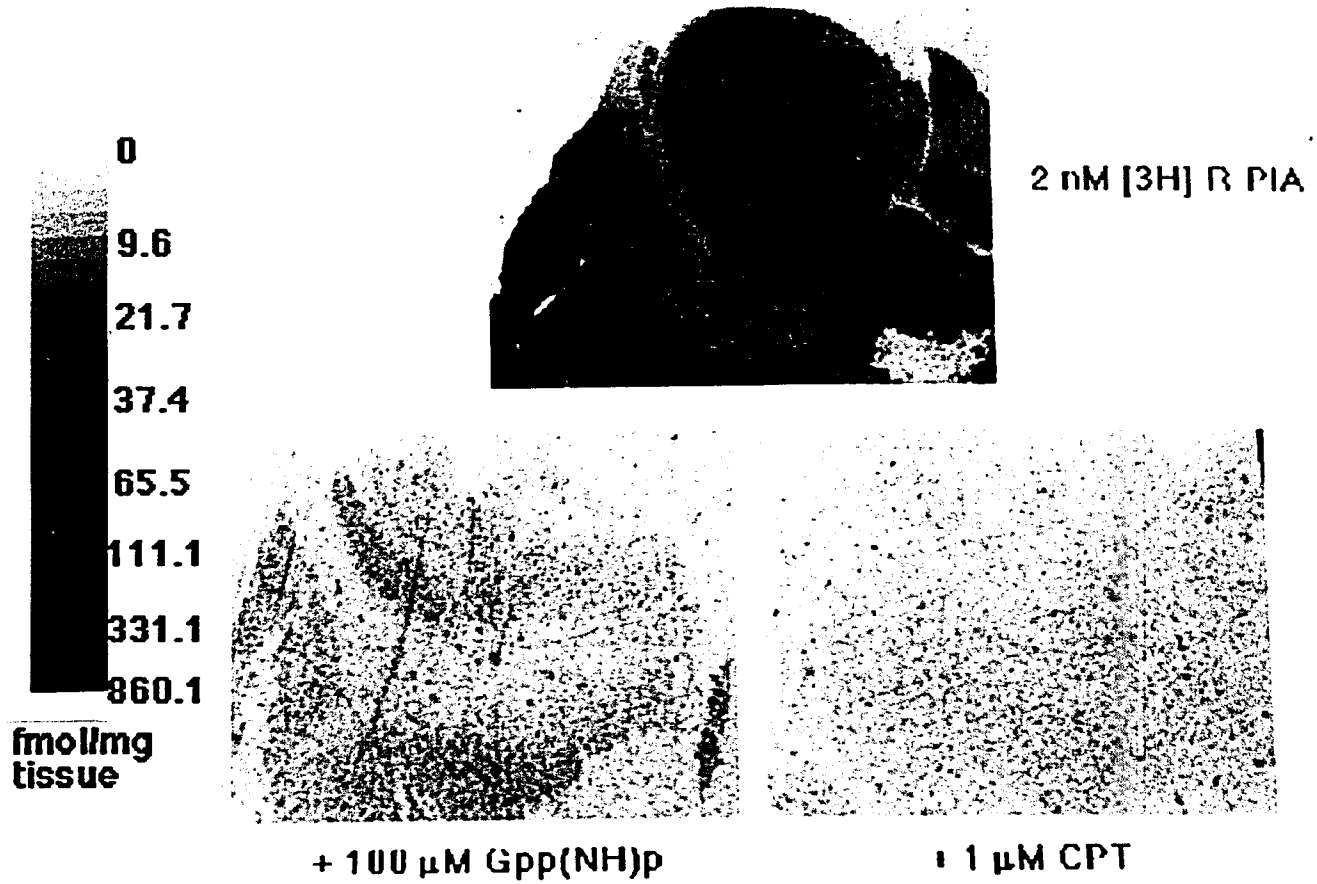

0
9.6
21.7
37.4
65.5
111.1
331.1
860.1
fmol/mg tissue
2 nM [3H] R PIA
+ 100 µM Gpp(NH)p
+ 1 µM CPT

Fig. 2. Attenuation of [^{3}H]-R-PIA binding to coronal sections of Fischer 344 rat brain at the level of dorsal hippocampus by Gpp(NH)p. Conditions of the assay are described in Materials and Methods. Top panel: [^{3}H]-R-PIA (2 nM) labeled 182 fmol/mg tissue in CA1 stratum radiatum and 113 fmol/mg tissue in the outer blade of the molecular layer of the dentate gyrus. Bottom left panel: Co-incubation of an adjacent section with [^{3}H]-R-PIA and 100 μM Gpp(NH)p resulted in binding of [^{3}H]-R-PIA to 16 and 14 fmol/mg tissue respectively. Bottom right panel: Co-incubation of [^{3}H]-R-PIA with 10 μM CPT reduced the binding of [^{3}H]-R-PIA uniformly to a background level. This experiment was repeated another two times generating similar results.

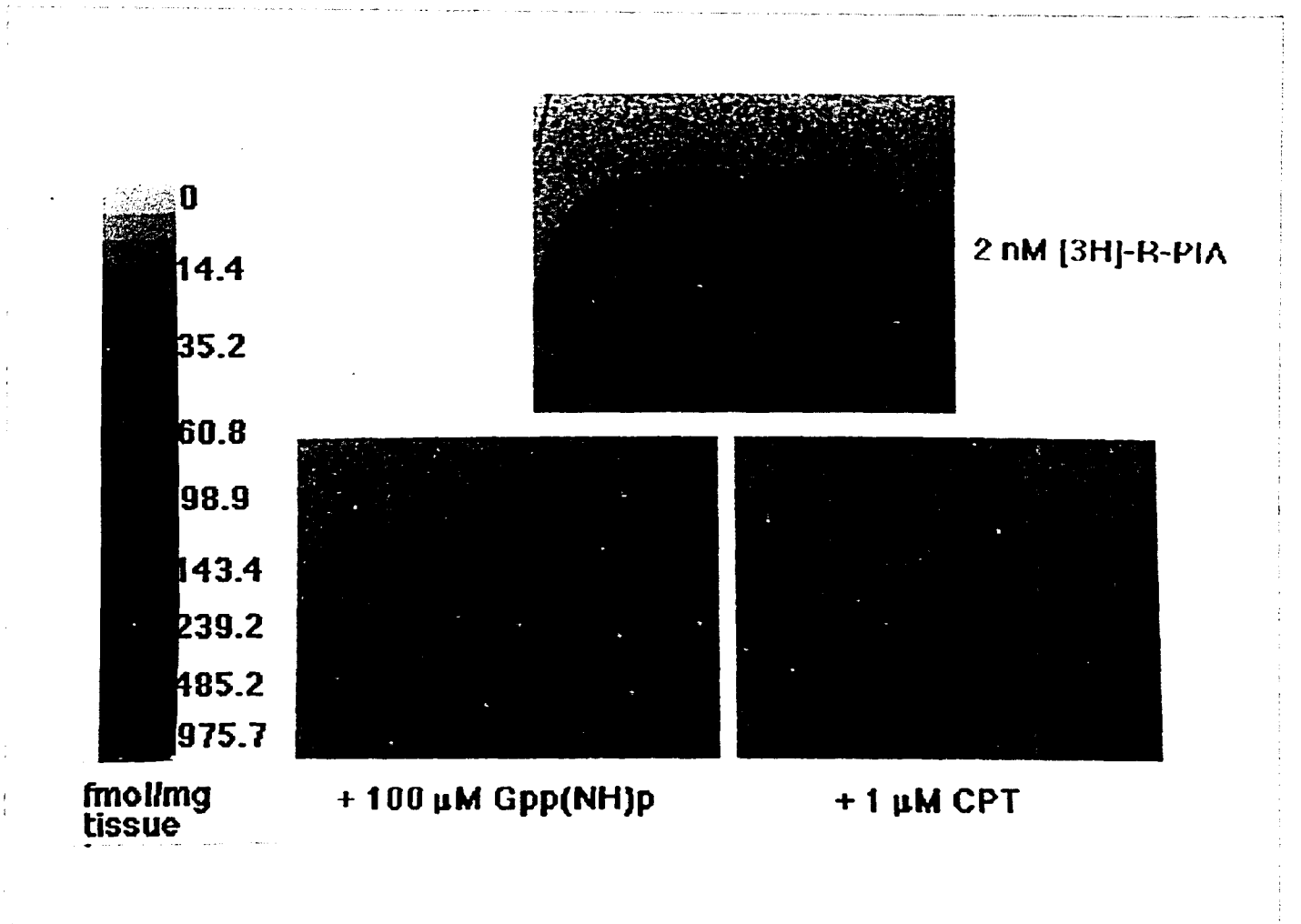

0
14.4
35.2
60.8
98.9
143.4
239.2
485.2
975.7
fmol/mg tissue
2 nM [3H]-R-PIA
+ 100 µM Gpp(NH)p
+ 1 µM CPT

Fig. 3. Attenuation of (±)-[^{3}H]-8-OH-DPAT binding to sections of human hippocampus by Gpp(NH)p. Conditions of the assay are described in Materials and Methods. Top Panel: (±)-[^{3}H]-8-OH-DPAT (2 nM) labeled 186 fmol/mg tissue in the CA1 stratum pyramidale, and 62 fmol/mg tissue in the dentate gyrus. Bottom left panel: Co-incubation of an adjacent section with (±)-[^{3}H]-8-OH-DPAT and 100 μM Gpp(NH)p resulted in binding of 17 and 8 fmol/mg tissue, respectively. Bottom right. Co-incubation of a third section with (±)-(±)-[^{3}H]-8-OH-DPAT and 1 μM BMY-7378 resulted in uniformly in background level binding. This experiment was repeated in another four human hippocampal sections, and showed similar results.

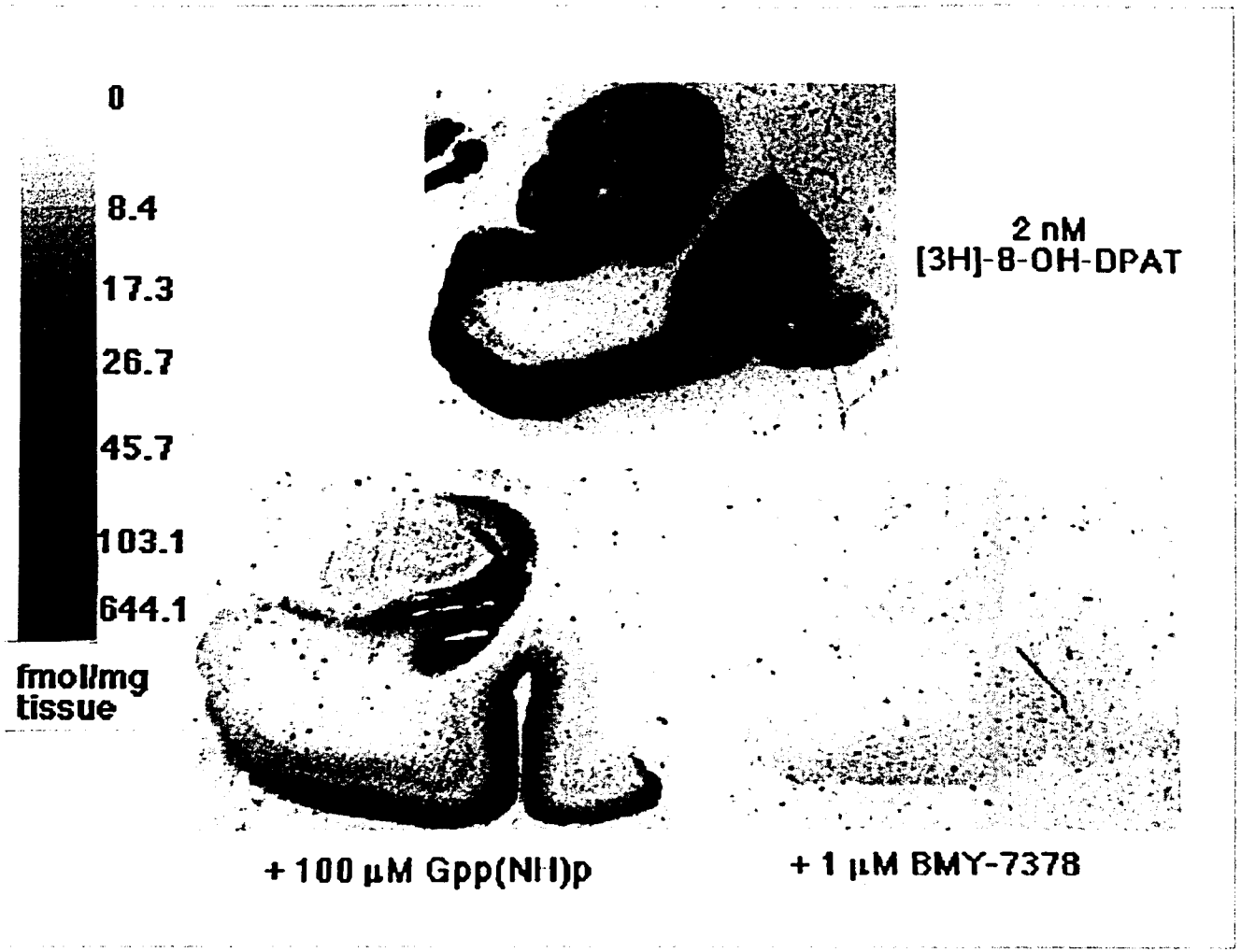

0
8.4
17.3
26.7
45.7
103.1
644.1
fmol/mg tissue
2 nM [3H]-8-OH-DPAT
+ 100 µM Gpp(NH)p
+ 1 µM BMY-7378

Fig. 4. Attenuation of (±)-[^{3}H]-8-OH-DPAT binding to coronal sections of rat brain at the level of dorsal hippocampus by Gpp(NH)p. Conditions of the assay are as described in Materials and Methods. Top panel: (±)-[^{3}H]-8-OH-DPAT (2 nM) labeled 154 fmol/mg tissue in CA1 stratum radiatum, and 189 fmol/mg tissue in the outer blade of the dentate gyrus. Bottom left panel: Co-incubation of an adjacent section with (±)-[^{3}H]-8-OH-DPAT in the presence of 100 μM Gpp(NH)p resulted in binding of 34 and 53 fmol/mg tissue, respectively. Bottom right panel: Co-incubation of a third adjacent section with (±)-[^{3}H]-8-OH-DPAT and 1 μM BMY-7378 resulted uniformly in a background level of binding. This experiment was repeated in three additional animals, producing similar results,

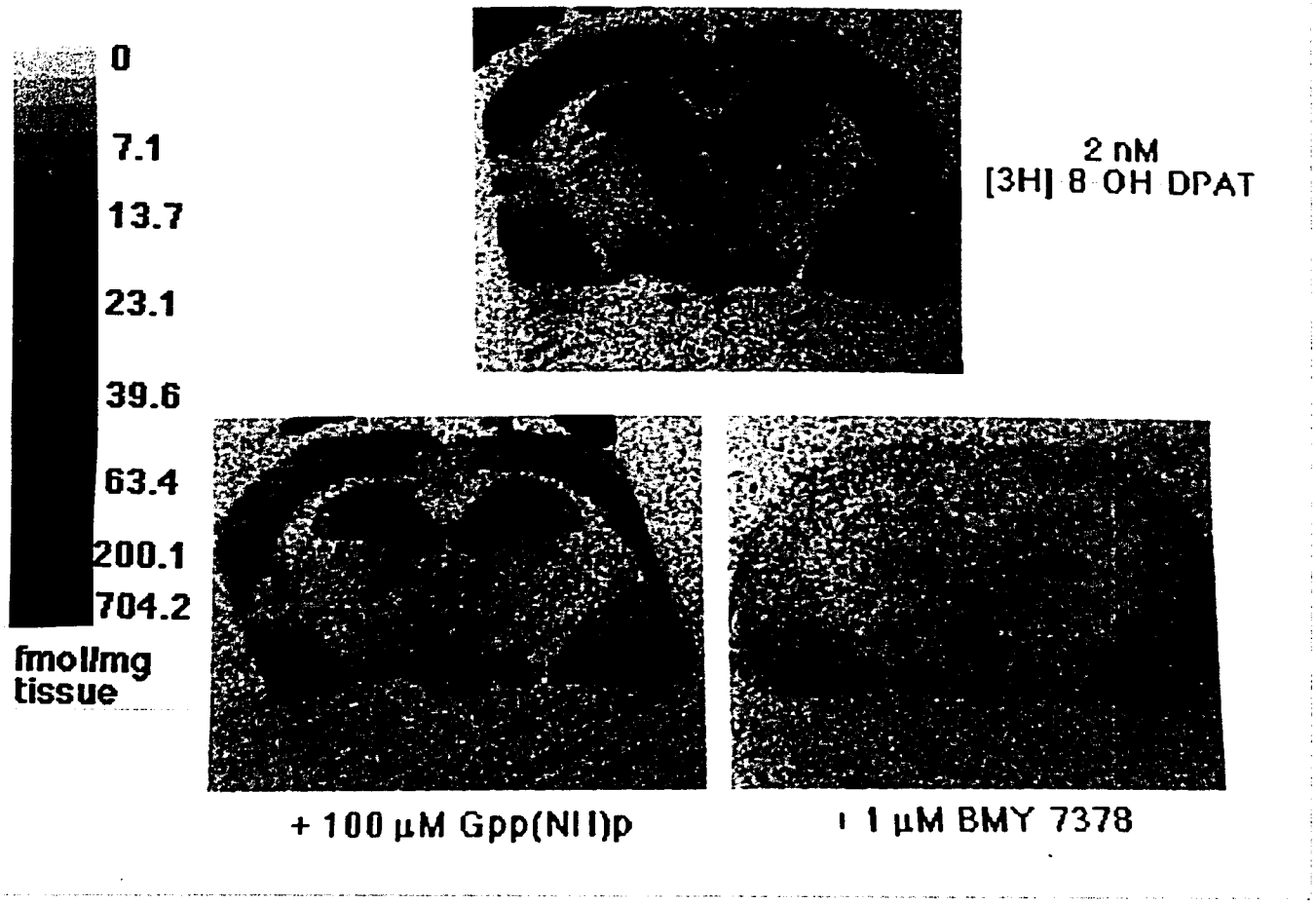
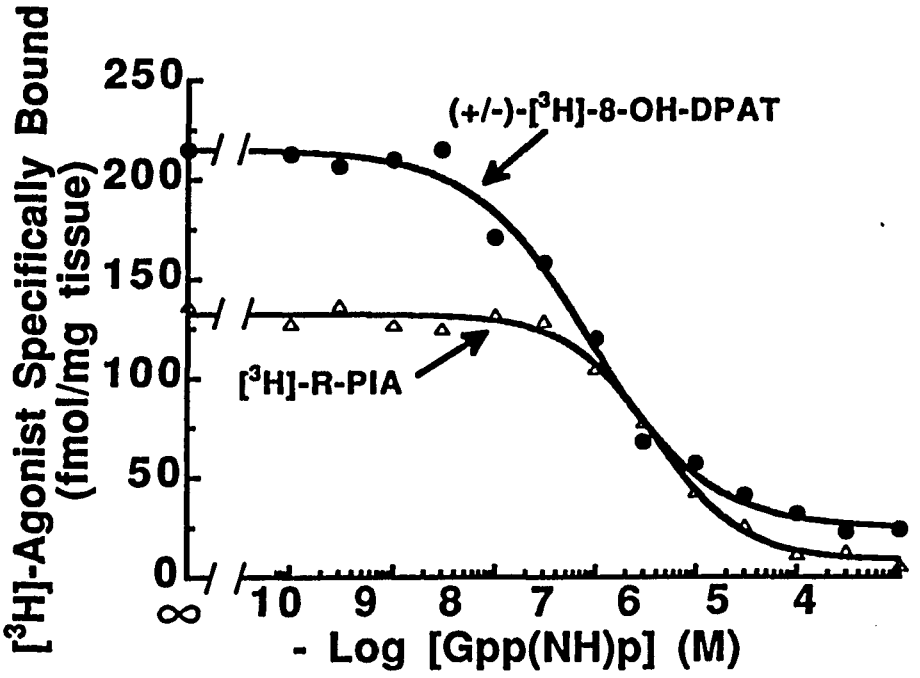

0
7.1
13.7
23.1
39.6
63.4
200.1
704.2
fmol/mg
tissue
2 nM
[3H] 8 OH DPAT
+ 100 µM Gpp(NH)p
+ 1 µM BMY 7378

Fig. 5. Illustration of concentration-effect curves of Gpp(NH)p attenuation of [^{3}H]-Agonist binding in CA1 stratum pyramidale from human hippocampus. Tissues were obtained and stored as described before (CHAPTER 2), and were prepared and assayed as described in Materials and Methods. Adjacent sections were incubated with 2 nM of either (±)-[^{3}H]-8-OH-DPAT or [^{3}H]-R-PIA, in the absence and the presence of 13 concentrations of Gpp(NH)p. Upper panel: As could be expected from reported K_d and B_{max} values in human hippocampal sections (CHAPTER 2), the similar percent occupancy of the receptor sites (80% by (±)-[^{3}H]-8-OH-DPAT and 73% by [^{3}H]-R-PIA) yielded a lower amount bound for the former (Table below). Points shown are means of quadruplicate determinations of the binding site density. Data were fit to a four parameter logistic equation (Materials and Methods), yielding the parameters shown below. Lower panel: Normalization of the data illustrates the consistently lower EC_{50} observed with the (±)-[^{3}H]-8-OH-DPAT binding. This experiment was repeated in an additional 4-5 different human hippocampal sections and curve parameters were determined in 6-10 regions (Tables 1 and 2).

[^{3}H]-Agonist	Initial Density (fmol/mg tissue)	EC_{50} (μM)	E_{max} (%)	slope index
(±)-[^{3}H]-8-OH-DPAT	216	0.086	88%	0.75
[^{3}H]-R-PIA	132	0.39	93%	0.99


[3H]-Agonist Specifically Bound
(fmol/mg tissue)
250
200
150
100
50
0
∞
(+/-)-[3H]-8-OH-DPAT
[3H]-R-PIA
10 9 8 7 6 5 4
- Log [Gpp(NH)p] (M)

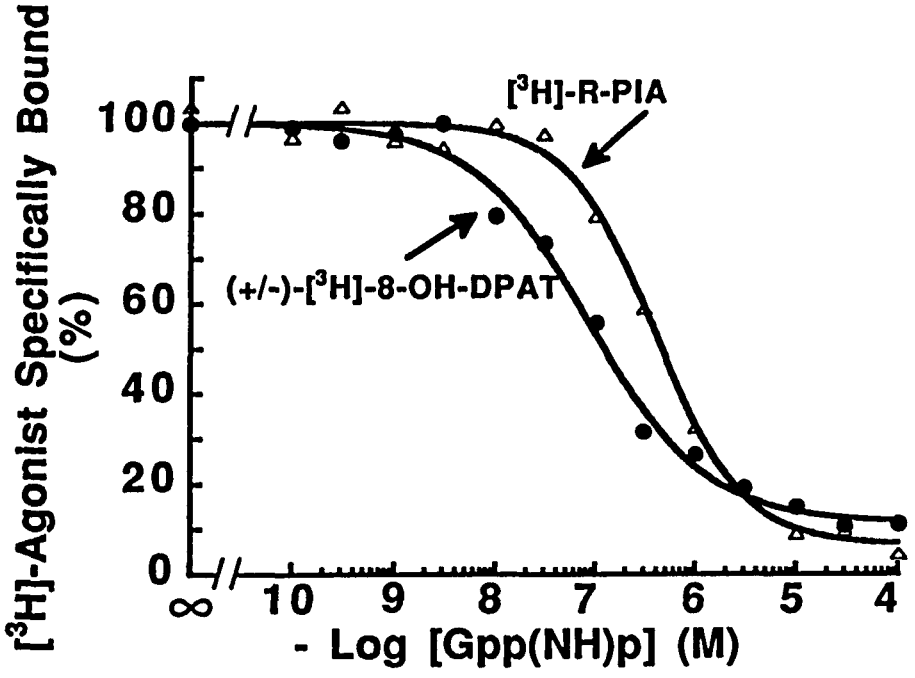
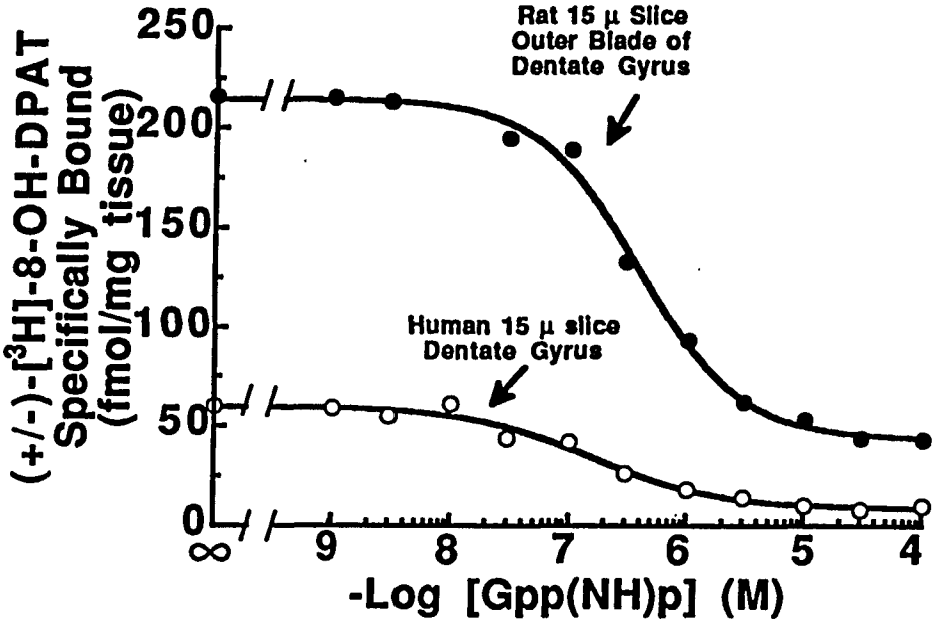

[3H]-Agonist Specifically Bound
(%)
100
80
60
40
20
0
∞
[3H]-R-PIA
(+/-)-[3H]-8-OH-DPAT
10 9 8 7 6 5 4
- Log [Gpp(NH)p] (M)

Fig. 6. Illustration of concentration-effect curves of Gpp(NH)p on (±)-[^{3}H]-8-OH-DPAT binding in sections of human hippocampus, CA1 stratum pyramidale or coronal sections of rat brain at the level of the dorsal hippocampus. Tissues were prepared and assayed as described in Materials and Methods. Adjacent sections were incubated with 2 nM (±)-[^{3}H]-8-OH-DPAT in the absence or presence of 13 concentrations of Gpp(NH)p. Upper panel: As could be expected from B_{max} and K_d values in the two species (CHAPTER 2), at the tested equal occupancy (87% and 84% in rat and human, respectively) and in the absence of Gpp(NH)p, the amount of bound (±)-[^{3}H]-8-OH-DPAT was higher in rat hippocampus (Table below). Shown are mean of quadruplicate determinations of receptor site density. Data were fit to a four parameter logistic equation (Materials and Methods), yielding the parameters shown below. Lower panel: normalization of the data illustrates the consistently lower EC_{50} values observed with the human sections. This experiment was repeated additional five times in human and 3 times in rat hippocampus, and curve parameters were calculated in 8-12 regions (Tables 2-3).

Species	Initial Density (fmol/mg tissue)	EC_{50} (μM)	E_{max} (%)	slope index
Human Dentate	61	0.16*	86%	0.82
Rat Outer Blade of Dentate	214	0.40	78%	1.00

*Statistically different ($p \leq 0.001$), from the rat value (n=4 rat, and 6 human). Other areas showing similar significant differences were, CA 1 stratum radiatum and lacunosum-moleculare.


(+/-)-[3H]-8-OH-DPAT Specifically Bound (fmol/mg tissue)
250
200
150
100
50
0
Rat 15 μ Slice Outer Blade of Dentate Gyrus
Human 15 μ slice Dentate Gyrus
∞
9
8
7
6
5
4
-Log [Gpp(NH)p] (M)

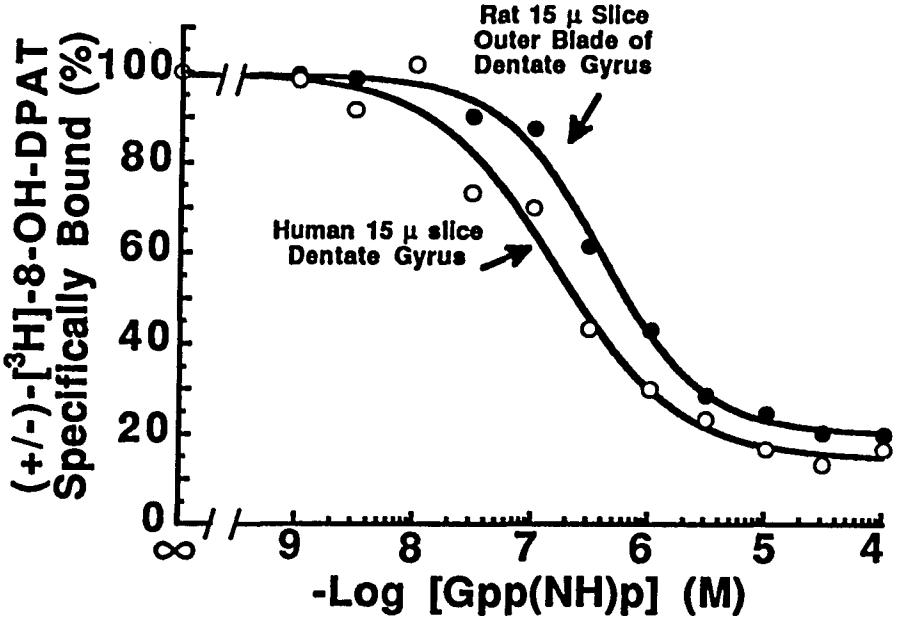

(+/-)-[3H]-8-OH-DPAT Specifically Bound (%)
100
80
60
40
20
0
Rat 15 μ Slice Outer Blade of Dentate Gyrus
Human 15 μ slice Dentate Gyrus
∞
9
8
7
6
5
4
-Log [Gpp(NH)p] (M)

Table 1

Parameters for the Attenuation of [^{3}H]-R-PIA Binding by Gpp(NH)p in Human Hippocampus

Experiments were performed as in Materials and Methods. Each number represents the mean + SEM of six human samples. For EC_{50} values, numbers in parentheses represent the arithmetic mean. For density, numbers in parentheses represent pmol/mg protein, METHODS, CHAPTER 2).

Region	Initial Density (fmol/mg tissue)	% Attenuation	pEC_{50}	slope index
CA1				
Stratum oriens	83±7 (0.83)	94.3±1.4	6.52 (0.35 μM)	1.32± 0.13
Stratum pyramidal	127±3 (1.27)	93.3±1.7	6.60 (0.32 μM)	1.47± 0.25
Stratum radiatum	117±8 (1.17)	94.7±1.2	6.60 (0.30 μM)	1.34± 0.22
Stratum lacunosum-moleculare	53±7 (0.53)	94.9±1.7	6.71 (0.27 μM)	1.32± 0.23
CA3				
Stratum oriens	91±6 (0.91)	94.5±1.7	6.82 (0.22 μM)	1.05± 0.08
Stratum pyramidale	70±6 (0.7)	93.3±1.8	6.96 (0.16 μM)	0.95± 0.07
Stratum radiatum	113±12 (1.13)	95.4±1.4	6.79 (0.20 μM)	1.09± 0.14
Stratum lacunosum-moleculare	62±3 (0.62)0	92.9±1.7	6.85 (0.19 μM)	0.97± 0.09
Ca4	69±8 (0.69)	93.3±1.2	6.92 (0.14 μM)	0.98± 0.09
Dentate	78±3 (0.78)	93.7±0.8	6.74 (0.23 μM)	1.05± 0.11
Cortex	92±2 (0.92)	92.3±2.0	6.44 (0.43 μM)	1.73± 0.40

Table 2

Parameters for the Attenuation of (±)-[^{3}H]-8-OH-DPAT Binding by Gpp(NH)p in Human Hippocampus

Experiments were performed as in Materials and Methods. Each number represents the mean + SEM of six human samples. For EC_{50} values, numbers in parentheses represent the arithmetic mean. For density, numbers in parentheses represent pmol/mg protein, METHODS, CHAPTER 2).

Region	Initial Density (fmol/mg tissue)	% Attenuation	pEC_{50}	slope index
CA1 Stratum oriens	96±8 (0.96)	89.6±2.1	6.70 (0.22 μM)	0.98± 0.11
Stratum pyramidal	194±7 (1.9)	91.0±1.0	6.80 (0.18 μM)	0.82± 0.10
Stratum radiatum	117±7 (1.17)	89.2±1.9	6.88 (0.14 μM)	1.06± 0.03
Stratum lacunosum-moleculare	119±6.3 (1.19)	89.4±1.8	6.92 (0.13 μM)	1.02± 0.05
Ca4	21±3 (0.21)	95.8±1.0	6.68 (0.22 μM)	0.94± 0.14
Dentate	46±5 (0.46)	91.1±2.0	6.91 (0.15 μM)	0.89± 0.13
Inner Layers of Cortex	24±5 (0.24)7	94.6±3.5	6.58 (0.48 μM)	1.01± 0.38
Outer Layers of Cortex	84±12 (0.84)	88.9±1.4	6.63 (0.32 μM)	1.09± 0.26

Table 3

Parameters for the Attenuation of (±)-[^{3}H]-8-OH-DPAT Binding by Gpp(NH)p in Rat Hippocampus

Experiments were performed as in Materials and Methods. Each number represents the mean + SEM of four separate rats. For EC_{50} values, numbers in parentheses represent the arithmetic mean. For density, numbers in parentheses represent pmol/mg protein, METHODS, CHAPTER 2).

Region	Initial Density (fmol/mg tissue)	% Attenuation	pEC_{50}	slope index
CA1				
Stratum oriens	98±8 (0.98)	79.4±2.4	6.50 (0.34 μM)	1.14± 0.13
Stratum radiatum	148±13 (1.5)	79.7±2.2	6.43 (0.37 μM)	1.02± 0.16
Stratum lacunosum moleculare	124±10 (1.24)	76.4±2.3	6.50 (0.31 μM)	0.80± 0.08
CA2				
Stratum oriens	15±3 (0.15)	86.5±5.9	6.51 (0.31 μM)	1.16± 0.05
Stratum pyramidale	10±2 (0.10)	95.6±2.4	6.51 (0.32 μM)	0.97± 0.21
CA3				
Stratum oriens	65±5 (0.65)	84.3±1.3	6.51 (0.26 μM)	1.07± 0.29
Stratum pyramidale	51±7 (0.51)	82.8±2.07	6.68 (0.26 μM)	0.93± 0.21
Dentate Gyrus				
Inner blade	146±14 (1.46)	72.5±0.8	6.51 (0.40 μM)	0.99± 0.12
Outer Blade	196±9 (1.96)	76.9±2.0	6.39 (0.41 μM)	0.96± 0.10

Table 3, continued.

Parameters for the Attenuation of (±)-[^{3}H]-8-OH-DPAT Binding by Gpp(NH)p in Rat Hippocampus (cont.)

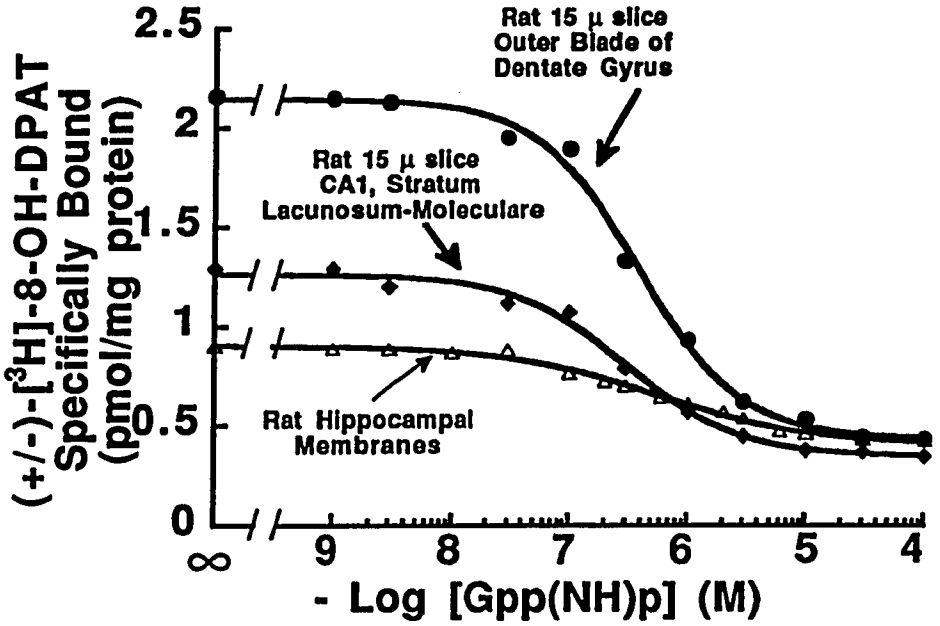
Region	Initial Density (pmol/mg protein)	% Attenuation	pEC_{50}	slope index
Hilus of Dentate	91±9 (0.91)	76.6±2.4	6.51 (0.45 μM)	0.89± 0.07
Hypothalamus	23±3 (0.23)	86.2±7.0	6.59 (0.26 μM)	0.80± 0.06
Cortex	31±6 (0.31)	84.3±4.2	6.52 (0.31 μM)	1.04± 0.12

Fig. 7. Illustration of concentration-effect curves of Gpp(NH)p on (±)-[^{3}H]-8-OH-DPAT binding in rat hippocampal membranes and 15 μ sections of rat dorsal hippocampus subregions. Upper panel: density of binding sites (pmol/mg protein) in the presence of increasing concentrations of Gpp(NH)p. Lower panel: normalization of data illustrates the difference in maximal attenuation in the 2 different preparations. Assuming a K_d value of 0.4 nM in both preparations (CHAPTERS 1 and 2), initial occupancy of binding sites was approximately 83%. Rat hippocampal membranes were prepared and used as described in CHAPTER 1 (Materials and Methods). The 15 μ coronal sections of rat brain were prepared and used as described in Materials and Methods. The concentration effect curves were analyzed using a four parameter logistic equation (Materials and Methods) which yielded the following parameters:

Preparation or Region	Initial Density (pmol/mg protein)***	EC_{50} (μM)	E_{max} (%)	slope index
Hippocampal Membranes	0.90	0.54	55%*	0.67
15 μ section CA1 S. lac-mol	1.27	0.30	73%	0.91
15 μ section dent. gyrus	2.14	0.40	79%	1.00

*Significantly different from all subregions measured using quantitative receptor autoradiography.

***For autoradiography density values (pmol/mg protein) were obtained as described in CHAPTER 2, METHODS.


(+/-)-[3H]-8-OH-DPAT Specifically Bound (pmol/mg protein)
2.5
2
1.5
1
0.5
0
Rat 15 μ slice Outer Blade of Dentate Gyrus
Rat 15 μ slice CA1, Stratum Lacunosum-Moleculare
Rat Hippocampal Membranes
∞ 9 8 7 6 5 4
- Log [Gpp(NH)p] (M)

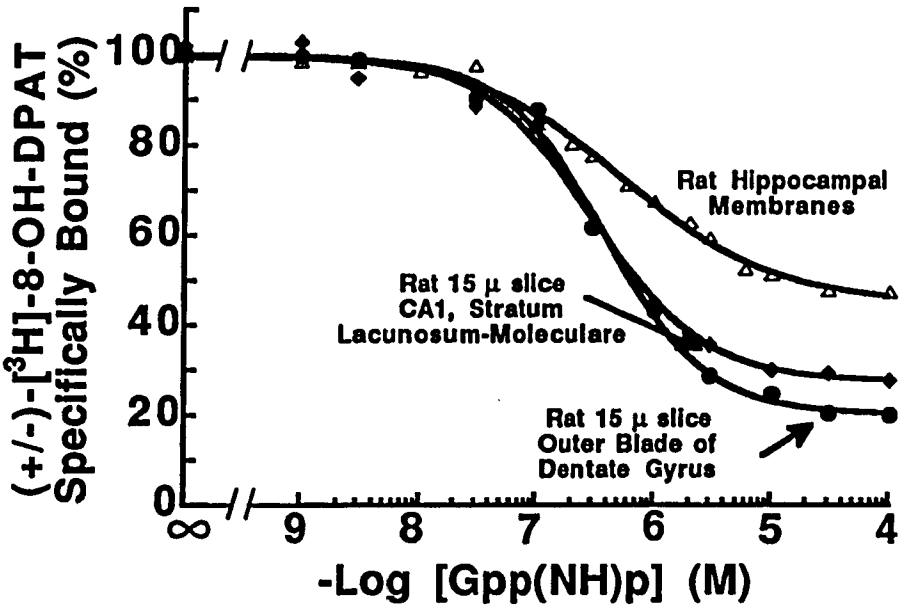

(+/-)-[3H]-8-OH-DPAT Specifically Bound (%)
100
80
60
40
20
0
Rat Hippocampal Membranes
Rat 15 μ slice CA1, Stratum Lacunosum-Moleculare
Rat 15 μ slice Outer Blade of Dentate Gyrus
∞ 9 8 7 6 5 4
-Log [Gpp(NH)p] (M)

Fig. 8. Kinetics of [^{3}H]-R-PIA association to AD A_1 receptor sites in post-mortem human hippocampus, CA1 stratum pyramidale, and of kinetics of dissociation elicited by CPT or Gpp(NH)p. All sections were pre-incubated for 15 min. in assay buffer (Materials and Methods), and incubated for 120 min. in [^{3}H]-R-PIA (2 nM). After 120 min. incubation, radioligand was removed, and 10 μM CPT or 100 μM Gpp(NH)p was added and radioligand replaced, and dissociation followed an additional 60 min. The density of binding at equilibrium was 200 fmol/mg tissue. Adjacent sections were preincubated in assay buffer as above, but co-incubated with 2 nM [^{3}H]-R-PIA in the presence of 10 μM CPT or 100 μM Gpp(NH)p. These sections generated values close to film background, 5 fmol/mg tissue for co-incubation with 10 μM CPT, and 9 fmol/mg tissue for co-incubation with Gpp(NH)p. After 60 min, the sections in which the ligand was dissociated by 10 μM CPT were reduced to 144 fmol/mg tissue, while those dissociated by 100 μM Gpp(NH)p displayed a density of 72 fmol/mg tissue.

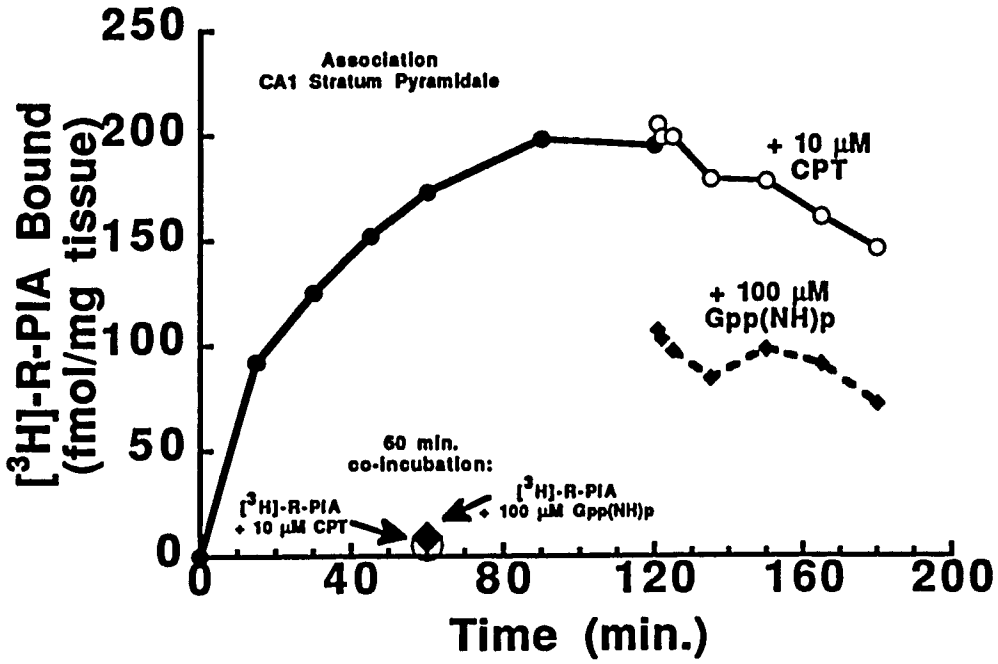

Association
CA1 Stratum Pyramidale
[3H]-R-PIA Bound
(fmol/mg tissue)
250
200
150
100
50
0
+ 10 µM
CPT
+ 100 µM
Gpp(NH)p
60 min.
co-incubation:
[3H]-R-PIA
+ 10 µM CPT
[3H]-R-PIA
+ 100 µM Gpp(NH)p
0
40
80
120
160
200
Time (min.)

Fig. 9. Kinetics of [^{3}H]-R-PIA dissociation. Upper panel left: [^{3}H]-R-PIA (2 nM) binding to AD A_1 receptor sites in post-mortem human hippocampus (120 min. incubation), middle panel: Adjacent section incubated in the presence of 10 μM CPT. Upper right panel: dissociation of [^{3}H]-R-PIA by 10 μM CPT, 60 min. after CPT. Lower left panel: Adjacent section co-incubated with [^{3}H]-R-PIA and 100 μM Gpp(NH)p. Lower right: dissociation of [^{3}H]-R-PIA by 100 μM Gpp(NH)p, after a prior incubation in [^{3}H]-R-PIA. Similar results were obtained in 15 μ sections of human cerebellum and rat dorsal hippocampus (data not shown). A complete time course illustrating this phenomenon is shown graphically in Fig. 8. Density of binding (fmol/mg tissue) in the CA1 stratum pyramidale and dentate gyrus, in each of the sections shown above, is listed below:

Region	control (120 min.)	non-specific binding	60 min. after dissociation by CPT	Co-incubation with Gpp(NH)p	60 min. after dissociation by Gpp(NH)p
S. Pyr.	273	13	133	14	45
Dent. Gyr.	171	1.5	69	9	28

Stabilization and Destabilization of the High Affinity Ternary Complex Can be Observed Kinetically

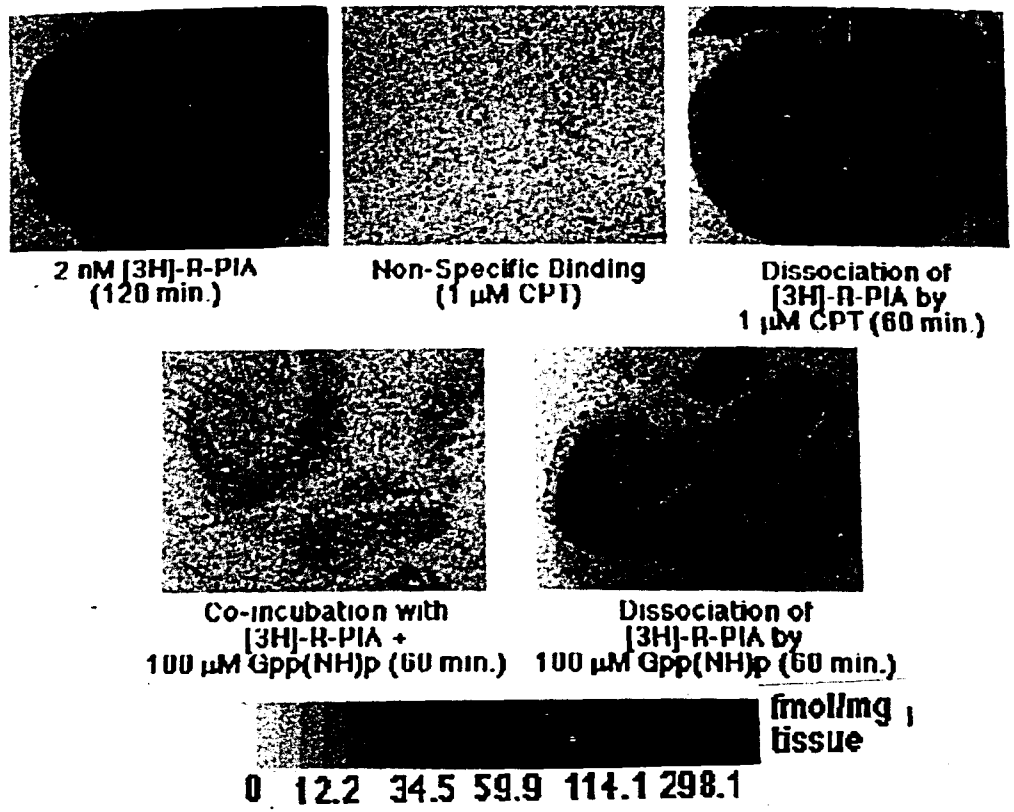


Fig. 10. Kinetics of (±)-[^{3}H]-8-OH-DPAT association to 5-HT$_{1A}$ receptor sites in post-mortem human hippocampus, CA1 stratum pyramidale, and kinetics of dissociation by elicited by BMY-7378 or Gpp(NH)p. All sections were pre-incubated for 15 min. in assay buffer (Materials and Methods), and incubated for 60 min. in (±)-[^{3}H]-8-OH-DPAT (2 nM). At equilibrium, the density of binding in CA1 stratum pyramidale was 292 fmol/mg tissue. After 60 min. incubation, radioligand was removed, and 1 μM BMY-7378 or 100 μM Gpp(NH)p was added and radioligand replaced, and the dissociation followed an additional 60 min. Adjacent sections were preincubated in assay buffer as above, but co-incubated with 2 nM (±)-[^{3}H]-8-OH-DPAT in the presence of 1 μM BMY-7378 or 100 μM Gpp(NH)p. These sections generated values close to film background, 2 fmol/mg tissue for co-incubation with 1 μM BMY-7378, and 18 fmol/mg tissue for co-incubation with Gpp(NH)p. After 60 min, the sections in which the ligand was dissociated by 1 μM BMY-7378 had approached film background, while sections dissociated by 100 μM Gpp(NH)p still displayed 69 fmol/mg tissue.

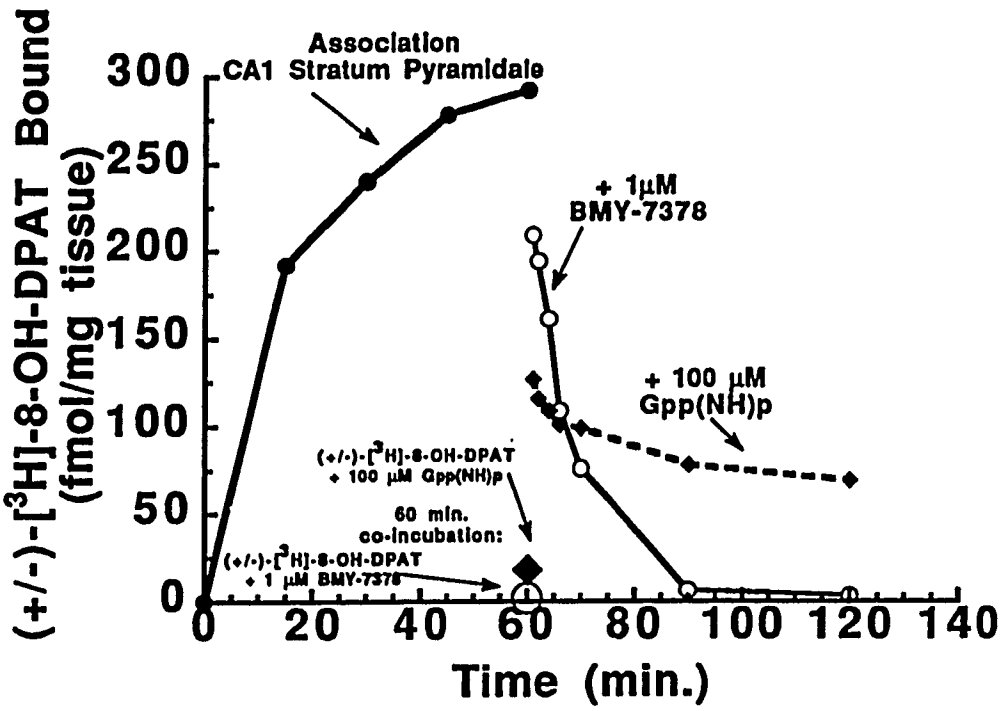
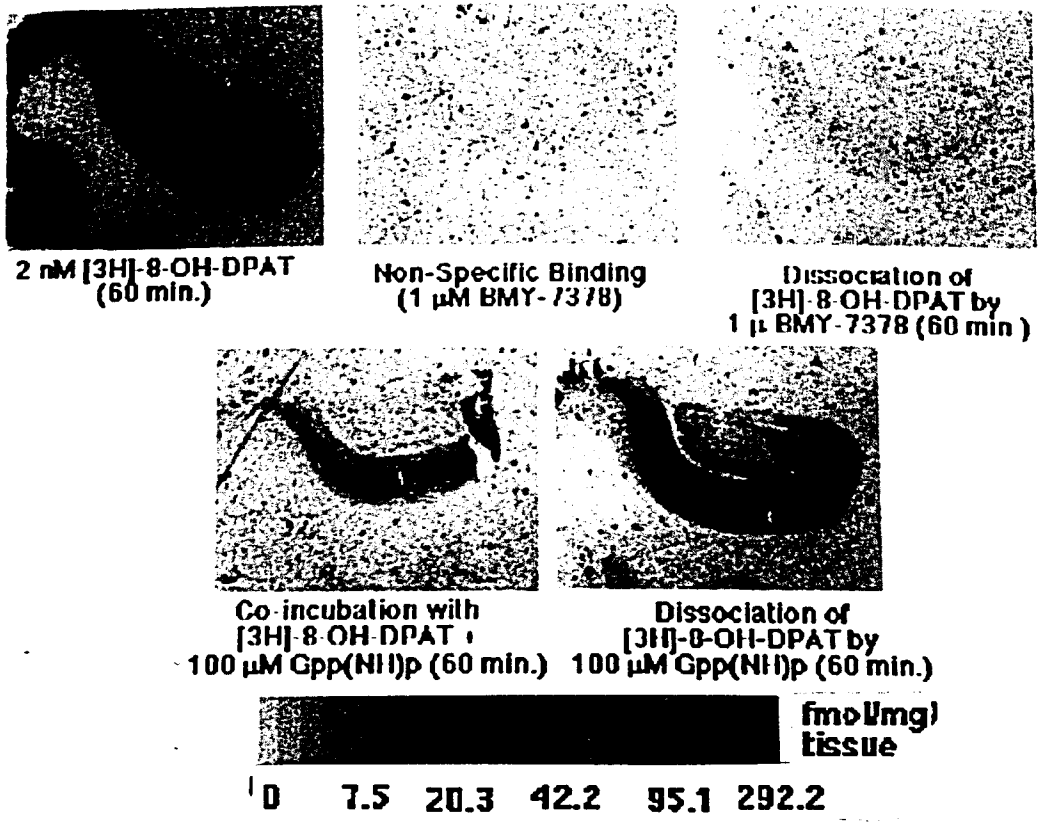

(+/-)-[3H]-8-OH-DPAT Bound (fmol/mg tissue)
300
250
200
150
100
50
0
Association
CA1 Stratum Pyramidale
+ 1μM BMY-7378
+ 100 μM Gpp(NH)p
(+/-)-[3H]-8-OH-DPAT + 100 μM Gpp(NH)p
60 min. co-incubation:
(+/-)-[3H]-8-OH-DPAT + 1 μM BMY-7378
0
20
40
60
80
100
120
140
Time (min.)

Fig. 11. Kinetics of (±)-[^{3}H]-8-OH-DPAT dissociation. Upper panel: (±)-[^{3}H]-8-OH-DPAT (2 nM) binding to the 5-HT_{1A} receptor sites in post-mortem human hippocampus after 60 min. incubation. Middle panel: Adjacent section incubated in the presence of 1 μM BMY-7378. Upper right panel: dissociation of (±)-[^{3}H]-8-OH-DPAT by 1 μM BMY-7378, 60 min. after addition of BMY-7378. Lower left panel: Adjacent section co-incubated with (±)-[^{3}H]-8-OH-DPAT and 100 μM Gpp(NH)p. Lower right: dissociation of (±)-[^{3}H]-8-OH-DPAT by 100 μM Gpp(NH)p, after a prior incubation in (±)-[^{3}H]-8-OH-DPAT. Similar results were obtained in 15 μ sections of rat dorsal hippocampus (data not shown). A complete time course illustrating this phenomenon is shown graphically in Fig. 10. The density of binding (fmol/mg tissue) in the CA1 stratum pyramidale and dentate gyrus, in each of the sections shown above, is listed below:

Region	control (120 min.)	non-specific binding	60 min. after dissociation by BMY-7378	Co-incubation with Gpp(NH)p	60 min. after dissociation by Gpp(NH)p
S. Pyr.	292	2	3	18	69
Dent. Gyr.	86	2	3	4	14

Stabilization and Destabilization of the High Affinity Ternary Complex Can be Observed Kinetically



DISCUSSION

The initial steps of receptor-mediated signal transduction in excitable cells require interaction between at least three proteins embedded in the cell membrane: receptor, guanine nucleotide binding protein (G-protein), and effector (an enzyme or channel). These proteins are referred to as a receptor system. Adenosine A_1 (AD A_1) and 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptors are coupled to K^+ channels and adenylyl cyclase, through activation of an inhibitory G-protein (G_i). Hormone binding to the receptor stabilizes a high affinity ternary complex formed between hormone/receptor/G-protein, H-R-G (Stadel et al., 1980). Guanine nucleotides (GN) rapidly destablize H-R-G, by conversion of the high affinity into the low affinity state. The ternary complex model was originally proposed for the G_s-linked beta-adrenergic receptor (De Lean, et al., 1980) and has since been proposed for other receptors coupled to effector systems through a variety of G-proteins, i.e., dopamine (Zahniser and Molinoff, 1978; Hamblin et al., 1984), 5-HT_1 (Schlegel and Peroutka, 1986; Stratford et al., 1988), 5-HT_2 (Strange, 1988; Strange, 1990), muscarinic (Ehlert, 1985, Galper et al., 1987), and opioid (Itzhak, 1989; Werling, et al., 1988). The allosteric interaction(s) between binding of the two ligands to the receptor system--hormone and GN-- was the subject of this study. The goal of the study was to identify characteristics of the allosteric interactions for two different receptor systems discretely localized in both rat and human brain using quantitative receptor autoradiography.

A reference system to the subsequent autoradiographic studies was rat hippocampal membranes, where parameters of the allosteric interactions were investigated with each of the two receptor systems. Three [^{3}H]-agonists were utilized: an AD A_1 agonist ([^{3}H]-R-PIA), and a full ((±)-[^{3}H]-8-OH-DPAT) and partial ([^{3}H]-BMY-7378) 5-HT_{1A} agonist. Initially, agonist/receptor related parameters were examined. The density (B_{max}, pmol/mg protein) of the H-R-G complex appeared to be dependent on both receptor type and agonist relative efficacy. Previously, for the beta-adrenergic receptor, density of ternary complex formed was related to agonist intrinsic activity (Kent et al., 1980). Accordingly, the greater amount of H-R-G destabilized into the low affinity state produced a greater functional response. GN destabilized H-R-G, and converted only half of these receptors into the low affinity state. This was quite surprising considering the large G-protein/receptor ratio proposed (Neubig et al., 1988). Due to the nature of the binding assay used, i.e., only the high affinity state measured, GN destabilization produced a decrease in the B_{max} value, with no change in the K_d value.

The effect of agonist binding on GN interaction was tested at the six agonist concentrations, where the initial receptor occupancy was increased four times (24-93%). In all cases GN attenuated [^{3}H]-agonist binding in a concentration-dependent and saturable manner. Parameters of the GN concentration-effect curves (EC_{50}, E_{max} and slope index) were used to characterize the allosterism occurring between binding of agonist to the receptor, and binding of GN to the G-protein(s). Similar EC_{50} values were observed for the two 5-HT_{1A}

agonists. In contrast, higher EC_{50} values were observed with the AD A_1 agonist at equivalent initial receptor occupancy. The EC_{50} values increased approximately four times with increasing receptors occupancy; EC_{50} (μM,): R-PIA 0.6-2.6; (±)-8-OH-DPAT and BMY-7378 0.18-0.8. These results indicate that drug relative efficacy does not display a relationship to an affinity-related parameter of GN for the receptor system. In contrast, the EC_{50} values were dependent on the receptor-system tested (AD A1 > 5-HT1A) reflecting possible variation between receptor-G-protein interactions across receptor system. A series of parallel studies were done with one of the effectors (adenylyl cyclase) linked to these receptor systems. The GTP-dependence of the inhibition of adenylyl cyclase activity was indistinguishable across the tested drugs: 0.3-0.5 μM. This observation supports the hypothesis that events related to drug relative efficacy may occur at the level of receptor-G-protein interaction and not at the GN/G-protein or G-protein/effector interaction. The other two parameters of the GN concentration-effect curves, E_{max} and slope index, also displayed drug and receptor dependence which may be related to the respective drug efficacy and to receptor/G-protein interactions.

Quantitative receptor autoradiography is an accepted procedure useful in mapping receptor distribution (Kuhar et al., 1986). Recently this procedure has been used to examine components of the receptor system distal to the receptor. [^{3}H]-Gpp(NH)p has been used to localized G-proteins (Gehlert and Wamsley, 1986), and [^{3}H]-forskolin used to localize adenylate cyclase

(McMartin and Summers, 1990, Zarbin et al., 1989). While currently these ligands do not address receptor-mediated signal transduction, they have been enormously useful in localization and evaluation of the relative density of these receptor system components. They have also successfully been used in determination of alterations associated with pathological conditions such as ischemia (Daval, et al., 1989b) and schizophrenia (Kerwin and Beats, 1990).

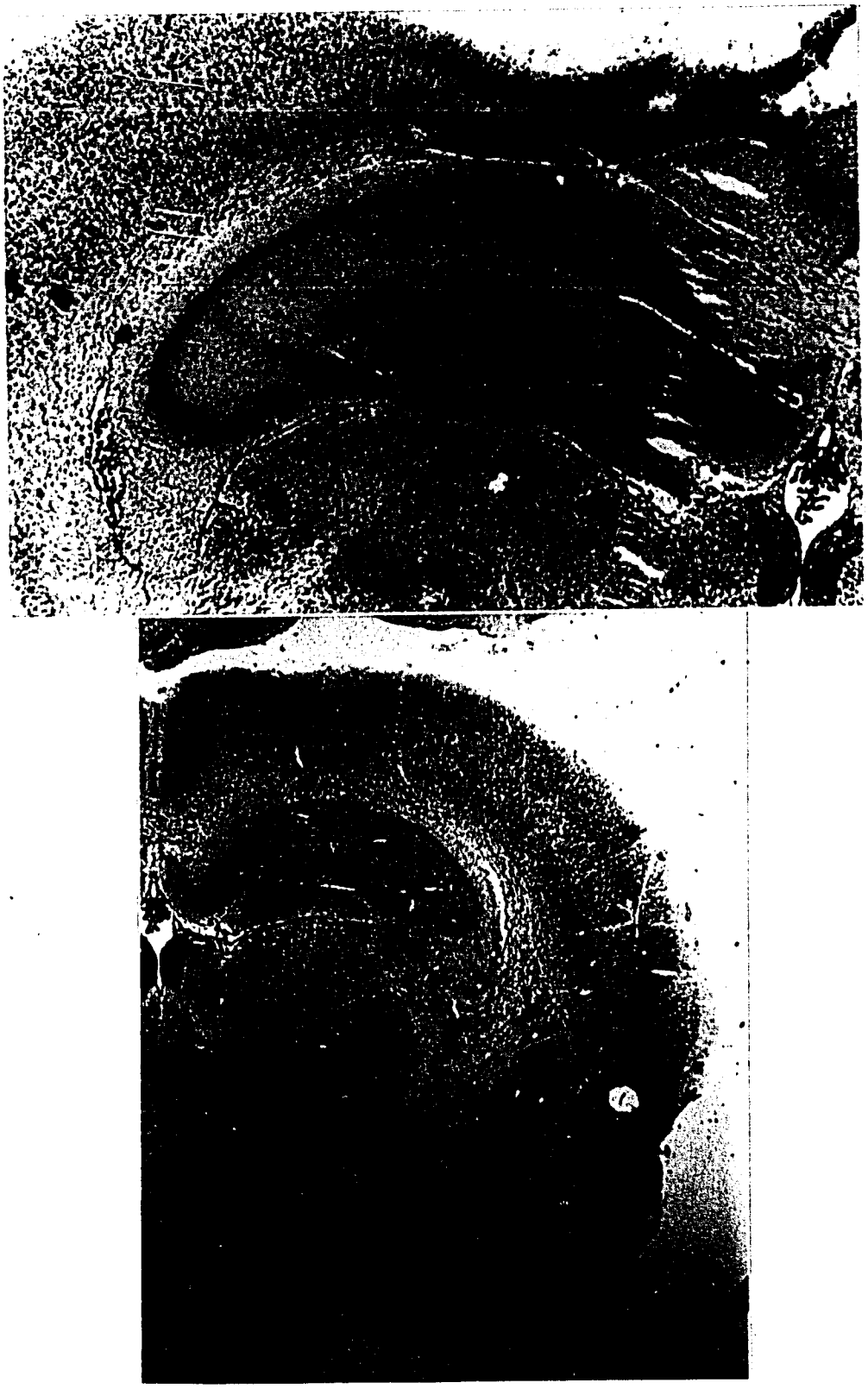
Receptor autoradiography was applied to study aspects of the allosteric interaction on the two different receptor systems in discrete hippocampal subregions of human and rat. Agonist affinity was similar across preparations in the rat (hippocampal membranes and subregions by QRA) and across the species, rat and human hippocampus: [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT K_d=0.5-1.0 nM. In contrast, density of [^{3}H]-agonist binding sites (B_{max}; pmol/mg protein) in rat hippocampus was increased in sections measured autoradiographically (CA1 stratum radiatum, pmol/mg protein; 3.6 and 1.7, [^{3}H]-R-PIA and (±)-[^{3}H]-8-OH-DPAT, respectively) when compared to a membrane preparation (1.44 and 1.04, respectively) The increase in B_{max} values obtained using QRA has been reported by others, for other receptors (Rainbow et al., 1984a; Rainbow et al., 1984b). This could be related to the measurement of defined regions which display the highest binding, as opposed to dilution high density regions by preparation of membrane homogenates. It has also been shown that polytronization of brain tissue causes a decrease in the density of binding sites (Norman et al., 1989).

Parameters of GN concentration-effect curves were determined autoradiographically in both rat and human brain hippocampal sections with a single concentration of each [^{3}H]-agonist (70-80% occupancy). In contrast to the consistent partial attenuation of [^{3}H]-agonist binding produced by saturating Gpp(NH)p concentrations in membrane preparations (50%); in brain sections from the two species (8-13 regions), we observed a near complete effect. Within regions, the EC_{50} values of the curves were indistinguishable for a given drug in a given species, but for (±)-[^{3}H]-8-OH-DPAT this parameter was invariably lower (0.1-0.2 μM) in human than in rat brain sections (0.3-0.4 μM) . Also, in human hippocampus the EC_{50} of the GN curves were lower for (±)-[^{3}H]-8-OH-DPAT than those of [^{3}H]-R-PIA (0.2-0.3 μM). The slope index of the curves did not differ from unity in any region, receptor, or species.

These studies have illustrated that allosteric interactions between binding of hormone and GN to receptor system can be characterized by parameters of the concentration attenuation curve in a membrane preparations. We have demonstrated that these parameters can be also measured reliably using quantitative receptor autoradiography, thus enabling a comparative anatomical study of different receptor systems across species. This approach to study the initial events of signal transduction across brain regions is complementary to methods in cell free systems from the brain.

APPENDIX

a. Cresyl violet stained section of human hippocampus.



b. Cresyl violet stained section of rat brain.

REFERENCES

Andersen, P. H. and J. A. Jansen. Dopamine receptor agonists: Selectivity and dopamine receptor efficacy. *Eur. J. Pharmacol. Mol. Pharmacol.* **188**: 335-347 (1990).

Andrade, R., R. C. Malenka and R. A. Nicoll. A G-protein couples serotonin and GABA B receptors to the same channels in hippocampus. *Science* **234**: 1261-1265 (1986).

Asarch, K. B. and J. C. Shih. Solubilization of serotonin $_{1A}$ and serotonin 1B binding sites from bovine brain. *J. Neurochem.* **48**: 1494-1501 (1987).

Barr, M. L. and J. A. Kiernan. The human nervous system: An anatomical viewpoint. (1983).

Birnbaumer, L. G-proteins in signal transduction. *Ann. Rev. Pharmacol. Toxicol.* **30**: 675-705 (1990).

Booze, R. M., E. A. Crisostomo and J. N. Davis. Species differences in the localization and number of CNS beta adrenergic receptors: Rat versus guinea pig. *J. Pharmacol. Exp. Ther.* **249**: 911-920 (1989).

Bradley, P. B., G. Engel, W. Feniuk, J. R. Fozard, P. P. A. Humphrey, D. N. Middlemiss, E. A. Mylecharane, B. P. Richardson and P. R. Saxena. Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology* **25**: 563-576 (1986).

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254 (1976).

Branchek, T., N. Adham, M. Macchi, H. T. Kao and P. R. Hartig. [^{3}H]-DOB (4-bromo-2,5-dimethoxyphenylisopropylamine) and [^{3}H]-ketanserin label two affinity states of the cloned human 5-hydroxytryptamine$_2$ receptor. *Mol. Pharmacol.* **38**: 604-609 (1990).

Bruns, R. F.. Adenosine receptor activation by adenine nucleotides requires conversion of the nucleotides to adenosine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **315**: 5-13 (1980).

Bruns, R. F., J. W. Daly and S. H. Snyder. Adenosine receptors in brain membranes: Binding of {N^6}- cyclohexyl[{3}H]adenosine and 1,3-diethyl-8-[{3}H]phenylxanthine. *Proc. Natl. Acad. Sci. USA* **77**: 5547-5551 (1980).

Casadó, V., C. Cantí, J. Mallol, E. I. Canela, C. Lluis and R. Franco. Solubilization of A_1 adenosine receptor from pig brain: Characterization and evidence of the role of the cell membrane on the coexistence of high- and low-affinity states. *J. Neurosci. Res.* **26**: 461-473 (1990).

Casey, P. J. and A. G. Gilman. G-protein involvement in receptor-effector coupling. *J. Biol. Chem.* **263**: 2577-2580 (1988).

Charney, D. S., J. H. Krystal, P. L. Delgado and G. R. Heninger. Serotonin-specific drugs for anxiety and depressive disorders. *Ann. Rev. Med.* **41**: 437-446 (1990).

Craddock-Royal, B., H. L. Wiener, and S. Maayani. Biochemical parameters associated with drug efficacy: Study of adenosine A_1 agonists by functional and binding assays in rat hippocampal membranes. *FASEB J.* **5**: A1572 (1991).

Dam, T. V. and R. Quirion. Pharmacological characterization and autoradiographic localization of substance P receptors in guinea pig brain. *Peptides* **7**: 855-864 (1986).

Daval, J. L., J. Deckert, S. R. B. Weiss, R. M. Post and P. J. Marangos. Upregulation of adenosine A_1 receptors and forskolin binding sites following chronic treatment with caffeine or carbamazepine: A quantitative autoradiographic study. *Epilepsia* **30**: 26-33 (1989a).

Daval, J. L., L. D. Von, J. Deckert, D. J. Redmond and P. J. Marangos. Protective effect of cyclohexyladenosine on adenosine A_1-receptors, guanine nucleotide and forskolin binding sites following transient brain ischemia: A quantitative autoradiographic study. *Brain Res.* **491**: 212-226 (1989b).

De Lean, A., J. M. Stadel and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**: 7108-7117 (1980).

De Vivo, M. and S. Maayani. Characterization of the 5-hydroxytryptamine$_{1A}$ receptor mediated inhibition of forskolin-stimulated adenylate cyclase activity in guinea pig and rat hippocampal membranes. *J. Pharmacol. Exp. Ther.* **238**: 248-253 (1986).

DeArmond, S. J., M. M. Fusco and M. M. Dewey. Structure of the human Brain: A photographic atlas. Oxford Press, New York (1976).

Dewar, D., K. Horsburgh, D. I. Graham, D. N. Brooks and J. McCulloch. Selective alterations of high affinity [^{3}H]forskolin binding sites in Alzheimer's disease: A quantitative autoradiographic study. *Brain Res.* **511**: 241-248 (1990).

Dragunow, M. and R. L. M. Faull. Neuroprotective effects of adenosine. *Trends Pharmacol. Sci.* **9**: 193-194 (1988).

Durcan, M. J. and P. F. Morgan. Prospective role for adenosine and adenosinergic systems in psychiatric disorders. *Psychol. Med.* **20**: 475-486 (1990).

Duvernoy, H. M.. The Human Hippocampus: An Atlas of Applied Anatomy. Springer-Verlag, New York (1988).

Ebersolt, C., A. Premont, A. Prochiantz, M. Perez and J. Bockaert. Inhibition of brain adenylate cyclase by A_1 adenosine receptors: Pharmacological characterization and locations. *Brain Res.* **267**: 123-129 (1983).

Ehlert, F. J.. The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. *Mol. Pharmacol.* **28**: 410-421 (1985).

El Mestikawy, S., C. Cognard, H. Gozlan and M. Hamon. Pharmacological and biochemical characterization of rat hippocampal 5-hydroxytrytpamine$_{1A}$ receptors solubilized by 3-[3=(cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS). *J. Neurochem.* **51**: 1031-1040 (1988).

Emerit, M. B., S. El Mestikawy, H. Gozlan, B. Rouot and M. Hamon. Physical evidence of the coupling of solubilized 5-HT$_{1A}$ binding sites with G regulatory proteins. *Biochem. Pharmacol.* **39**: 7-18 (1990).

Erfurth, A. and M. Reddington. Properties of binding sites for [^{3}H]-Cyclohexyladenosine in the hippocampus and other regions of rat brain: A quantitative autoradiographic study. *Neurosci. Lett.* **64**: 116-120 (1986).

Fargin, A. , J. R. Raymond, M. J. Lohse, B. K. Kobilka, M. G. Caron and R. J. Lefkowitz. The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT 1A receptor. *Nature* **335**: 358-360 (1988).

Fargin, A., J. R. Raymond, J. W. Regan, S. Cotecchia, R. J. Lefkowitz and M. G. Caron. Effector coupling mechanisms of the cloned 5-HT_{1A} receptor. *J. Biol. Chem.* **264**: 14848-14852 (1989).

Fastbom, J. and B. B. Fredholm. Regional differences in the GTP-dependence of adenosine receptor binding in rat brain. *Acta Physiol. Scand.* **131**: 467-470 (1987).

Fastbom, J. and B. B. Fredholm. Effects of long-term theophylline treatment on adenosine A_1-receptors in rat brain: Autoradiographic evidence for increased receptor number and altered coupling to G-proteins. *Brain Res.* **507**: 195-199 (1990a).

Fastbom, J. and B. B. Fredholm. Regional differences in the effect of guanine nucleotides on agonist and antagonist binding to adenosine A_1-receptors in rat brain, as revealed by autoradiography. *Neuroscience* **34**: 759-769 (1990b).

Fastbom, J., A. Pazos and J. M. Palacios. The distribution of adenosine A_1 receptors and 5'-nucleotidase in the brain of some commonly used experimental animals. *Neuroscience* **22**: 813-826 (1987a).

Fastbom, J., A. Pazos, A. Probst and J. M. Palacios. Adenosine A_1 receptors in the human brain: a quantitative autoradiography study. *Neuroscience* **22**: 827-840 (1987b).

Feller, D. J. and D. B. Bylund. Comparison of alpha2 adrenergic receptors and their regulation in rodent and porcine species. *J. Pharmacol. Exp. Ther.* **228**: 275-282 (1984).

Ferkany, J. W., H. L. Valentine, G. A. Stone and M. W. Williams. Adenosine A_1 receptors in mammalian brain: Species differences in their interactions with agonists and antagonists. *Drug Dev. Res.* **9**: 85-93 (1986).

Frazer, A. and J. G. Hensler. 5-HT1A receptors and 5-HT_{1A}-mediated responses: Effect of treatments that modify serotonergic neurotransmission. *Ann. N.Y. Acad. Sci.* **600**: 460-475 (1990).

Fredholm, B. B., B. Jonzon and K. Lindstrom. Effect of adenosine receptor agonists and other compounds on cyclic AMP accumulation in forskolin-treated hippocampal slices. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **332**: 173-178 (1986).

Galitzky, J., M. Lafontan, H. Paris and M. Berlan. Human fat cell alpha-2 adrenoceptors. II. Comparative study of partial and full agonist binding parameters using [^{3}H]-clonidine and [^{3}H]-UK-14,304. *J. Pharmacol. Exp. Ther.* **249**: 592-600 (1989).

Galper, J. B., L. S. Haigh, A. C. Hart, D. S. O'Hara and D. J. Livingston. Muscarinic cholinergic receptors in the embryonic chick heart: Interaction of agonist, receptor, and guanine nucleotides studied by an improved assay for direct binding of the muscarinic agonist [^{3}H]-0cismethyldioxolane. *Mol. Pharmacol.* **32**: 230-240 (1987).

Gavish, M., R. R. Goodman and S. H. Snyder. Solubilized adenosine receptors in the brain: Regulation by guanine nucleotides. *Science* **215**: 1633-1635 (1982).

Geary, W. A., A. W. Toga and G. F. Wooten. Quantitative film autoradiography for tritium: Methodological considerations. *Brain Res.* **337**: 99-108 (1985).

Gehlert, D. R. and J. K. Wamsley. In vitro autoradiographic localization of guanine nucleotide binding sites in sections of rat brain labeled with [^{3}H]guanylyl-5'imidodiphosphate. *Eur. J. Pharmacol.* **129**: 169-174 (1986).

Gerwins, P., C. Nordstedt and B. B. Fredholm. Characterization of adenosine A1 receptors in intact DDT1 MF-2 smooth muscle cells. *Mol. Pharmacol.* **38**: 660-666 (1990a).

Gerwins, P., C. Norstedt and B. B. Fredholm. GTP and IAP shifts adenosine A_1-agonist binding in smooth muscle membranes to the low affinity state, similar to the one found in intact cells. *Eur. J. Pharmacol.* **183**: 2137 (1990b).

Gilman, A. G.. G proteins: transducers of receptor-generating signals. *Ann. Rev. Biochem.* **56**: 615-649 (1987).

Glaser, T., M. Rath, J. Traber, K. Zilles and A. Schleicher. Autoradiographic identification and topographical analysis of high affinity serotonin receptor subtypes as a target for the novel putative anxiolytic TVXQ 7821. *Brain Res.* **358**: 129-136 (1985).

Glennon, R. A., N.A. Naiman, M. E. Pierson, M. Titeler, R.A. Lyon, and E. Weisberg. NAN-190: an arylpiperazine analog that antagonizes the stimulus effects of the 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT). *Eur. J. Pharmacol.* **154**: 339 (1988).

Goodman, R. R. and S. H. Snyder. Autoradiographic localization of adenosine receptors in rat brain using [^{3}H]cyclohexyladenosine. *J. Neurosci.* **2**: 1230-1241 (1982).

Gozlan, H., S. El Mestikawy, L. Pichat, J. Glowinski and M. Hamon. Identification of presynaptic serotonin autoreceptors using a new ligand:^{3}H- PAT. *Nature* **305**: 140 (1983).

Hall, M. D, S. El Mestikawy, M. B. Emerit, L. Pichat, M. Hamon and H. Gozlan. [^{3}H]-8-Hydroxy-2-(Di-n-Propyl)Tetralin Binding to Pre-and Postsynaptic 5-hydroxytryptamine sites in various regions of the rat brain. *J. Neurochem.* **44**: 1685-1696 (1985).

Hamblin, M. W., S. L. Leff and I. Creese. Interactions of agonists with D-2 dopamine receptors: evidence for a single receptor population existing in multiple agonist affinity states in rat striatal membranes. *Biochem. Pharmacol.* **33**: 877-887 (1984).

Harrington, M. A., D. Oksenberg and S. J. Peroutka. 5-Hydroxytryptamine $_{1A}$ receptors are linked to a G_i -adenylate cyclase complex in rat hippocampus. *Eur. J. Pharmacol.* **154**: 95-98 (1988).

Harrington, M. A. and S. J. Peroutka. Modulation of 5-hydroxytryptamine$_{1A}$ receptor density by nonhydrolyzable GTP analogues. *J. Neurochem.* **54**: 294-299 (1990).

Helmke, S. M. and D. Cooper. Solubilization of stable adenosine A_1 receptors from rat brain. *Biochem. J.* **257**: 413-418 (1989).

Herrick-Davis, K. and M. Titeler. [^{3}H]Spiroxatrine: a 5-HT$_{1A}$ radioligand with agonist binding properties. *J. Neurochem.* **50**: 528-533 (1988).

Heuring, R. E. and S. J. Peroutka. Characterization of a novel [^{3}H]-5-Hydroxytryptamine binding site subtype in bovine brain membranes. *J. Neurosci.* **7**: 894-903 (1987).

Heuring, R. E., J. R. Schlegel and S. J. Peroutka. Species variations in RU 24969 interactions with non-5HT$_{1A}$ binding sites. *Eur. J. Pharmacol.* **122**: 279-282 (1986).

Hoyer, D. and D. N. Middlemiss. Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. *Trends Pharmacol. Sci.* **10**: 130-132 (1989).

Hoyer, D., A. Pazos, A. Probst and J. M. Palacios. Serotonin receptors in the human brain. I. Characterization and autoradiographic localization of 5-HT$_{1A}$ recognition sites. Apparent absence of 5-HT$_{1B}$ recognition sites. *Brain Res.* **376**: 85-96 (1986).

Humphrey, P., W. Feniuk, M. J. Perren, I. Beresford, M. Skingle and E. T. Whalley. Serotonin and migraine. *Ann. N.Y. Acad. Sci.* **600**: 587-600 (1990).

Hunter, A. J.. Colloquium: Alzheimer's disease. Serotonergic involvement in learning and memory. *Biochem. Soc. Trans.* **17**: 79-81 (1989).

Itzhak, Y.. Multiple affinity binding states of the sigma receptor: Effect of GTP-binding protein-modifying agents. *Mol. Pharmacol.* **36**: 512-517 (1989).

Jagadeesh, G., E. J. Cragoe and R. C. Deth. Modulation of bovine aortic alpha-2 receptors by Na+, 5'-guanylylimidodiphosphate, amiloride, and ethylisopropylamiloride: evidence for receptor G-protein precoupling. *J. Pharmacol. Exp. Ther.* **252**: 1184-1196 (1990).

Jakobs, K. H., W. Sauer and G. Schultz. Characterization of α and β receptors linked to human platelet adenylate cyclase. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **302**: 285-291 (1978).

Januszewicz von Lubitz, D. K. E., J. M. Dambrosia and D. J. Redmond. Protective effect of cyclohexyl adenosine in treatment of cerebral ischemia in gerbils. *Neuroscience* **30**: 451-462 (1989).

Jarvis, M. F. and M. Williams. Direct autoradiographic localization of adenosine A2 receptors in the rat brain using the A_2-selective agonist, [^{3}H]-CGS 21680. *Eu.r J. Pharmacol.* **168**: 243-246 (1989).

Kenakin, T. P.. Pharmacologic Analysis of Drug-Receptor Interaction. Raven Press, New York (1987).

Kent, R. S., A. De Lean and R. J. Lefkowitz. A Quantitative analysis of Beta-adrenergic receptor interactions: Resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. *Mol. Pharmacol.* **17**: 14-23 (1980).

Kerwin, R. W. and B. C. Beats. Increased forskolin binding in the left parahippocampal gyrus and CA1 region in post mortem schizophrenic brain determined by quantitative autoradiography. *Neurosci. Lett* **118**: 164-168 (1990).

Kline, T., H. Park and L. R. Meyerson. CHAPS solubilization of a G-protein sensitive 5-HT_{1A} receptor from bovine hippocampus. *Life Sci.* **45**: 1997-2005 (1989).

Klotz, K.-N. and M. Lohse. The glycoprotein nature of A_1 adenosine receptors. *Biochem. Biophys. Res. Commun.* **140**: 406-413 (1986).

Klotz, K.-N., M. J. Lohse and U. Schwabe. Characterization of the solubilized A_1 adenosine receptor from rat brain membranes. *J. Neurochem.* **46**: 1528-1534 (1986).

Klotz, K.- N., M. J. Lohse, U. Schwabe, G. Cristalli, S. Vittori and M. Grifantini. 2-Chloro-N6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA)-A high affinity agonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**: 679-683 (1989).

Kohler, C., A.-C. Radesater, W. Lang and V. Chan-Palay. Distribution of serotonin-1A receptors in the monkey and the postmortem human hippocampal region. A quantitative autoradiographic study using the selective agonist [^{3}H]8-OH-DPAT. *Neurosci. Lett.* **72**: 43-48 (1986).

Kolassa, N., K. D. Beller and K. H. Sanders. Evidence for the interaction of urapidil with 5-HT_{1A} receptors in the brain leading to a decrease in blood pressure. *Am. J. Cardiol.* **63**: 36C-39C (1989).

Kuhar, M. J., E. B. DeSouza and J. R. Unnerstall. Neurotransmitter receptor mapping by autoradiography and other methods. *Ann. Rev. Neurosci.* **9**: 27-59 (1986).

Lee, K. S., P. Schubert, M. Reddington and G. W. Kreutzberg. The distribution of adenosine A_1 receptors and 5'-nucleotidase in the hippocampal formation of several mammalian species. *J. Comp. Neurol.* **246**: 427-434 (1986).

Lefkowitz. R. J., L. E. Limbird, C. Mukherjee and M. G. Caron. The β-adrenergic receptor and adenylate cyclase. *Biochim. Biophys. Acta* **457**: 1-39 (1976a).

Lefkowitz, R. J., D. Mullikin and M. G. Caron. Regulation of β-adrenergic receptors by guanyl-5'-ylimidodiphosphate and other purine nucleotides. *J. Biol. Chem.* **251**: 4686-4692 (1976b).

Leibowitz, S. F.. The role of serotonin in eating disorders. *Drugs* **39**: 33-48 (1990).

Leysen, J. E., J. E. Niemegeers, J. M. Van Neuten and P. M. Laduron. [^{3}H]-Ketanserin (R41 468), a selective [^{3}H]-ligand for serotonin$_2$ receptor binding sites. *Mol. Pharmacol.* **21**: 301-314 (1982).

J. Linden. Adenosine deaminase for removing adenosine: How much is enough? *Trends Pharmacol. Sci.* **10**: 260-262 (1989).

Lohse, M. J., K.-N. Klotz, J. Lindenborn-Fotinos, M. Reddington, U. Schwabe and R. A. Olsson. 8-Cyclophentyl-1,3-dipropylxathine (DPCPX) - a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336**: 204-210 (1987).

Lohse, M. J., K.-N. Klotz and U. Schwabe. Agonist photoaffinity labeling of A_1 adenosine receptors: Persistent activation reveals spare receptors. *Mol. Pharmacol.* **30**: 403-409 (1986).

Lohse, M. J., V. Lenschow and U. Schwabe. Two affinity states of R_i adenosine receptors in brain membranes. Analysis of guanine nucleotide and temperature effects on rdioligand binding. *Mol. Pharmacol.* **26**: 1-9 (1984).

Lyon, R. A., K. H. Davis and M. Titeler. ^{3}H-DOB (4-bromo-2,5,-dimethoxyphenylisopropylamine) labels a guanyl nucleotide-sensitive state of cortical 5-HT_2 receptors. *J. Pharmacol. Exp. Ther.* **31**: 194-199 (1987).

Mandal, A. K., K. J. Kellar, E. Friedman, S. V. Pineo, P. Hamosh and R. A. Gillis. Importance of central nervous system serotonin-$_{1A}$ receptors for mediating the hypotensive effect of urapidil. *J. Pharmacol. Exp. Ther.* **251**: 563-570 (1989).

Marcinkiewicz, M., D. Verge, H. H. Gozlan, L. Pichat and M. Hamon. Autoradiographic evidence for the heterogeneity of 5-HT_1 sites in the rat brain. *Brain Res.* **291**: 159-163 (1984).

Martens, D., M. J. Lohse and U. Schwabe. [^{3}H]-8-Cyclopentyl-1,3-dipropylxanthine binding to A1 receptors of intact rat ventricular myocytes. *Circ. Res.* **63**: 613-620 (1988).

Martial, J., S. Lal, M. Dalpé, A. Olivier, M. C. De and R. Quirion. Apparent absence of serotonin$_{1B}$ receptors in biopsied and post-mortem human brain. *Synapse* **4**: 203-209 (1989).

McMartin, L. R. and R. J. Summers. The autoradiographic localization of adenylate cyclase in rat kidney using [^{3}H]forskolin. *Biochem. Pharmacol.* **39**: 1019-1028 (1990).

Mendelson, S. D. and B. B. Gorzalka. 5-HT_{1A} receptors: differential involvement in female and male sexual behavior in the rat. *Physiol. Behav.* **37**: 345-351 (1986).

Miyoshi, R. and S. Kito. Ontogeny of phorbol ester receptors in rat brain studied by in vitro autoradiography. *J. Neural. Transm.* **81**: 41-51 (1990).

Munshi, R. and J. Linden. Co-purification of A_1 adenosine receptors and guanine nucleotide-binding proteins from bovine brain. *J. Biol. Chem.* **264**: 14853-14859 (1989).

Murphy, K. M. M. and S. H. Snyder. Heterogeneity of Adenosine A_1 Receptor Binding in Brain Tissue. *Mol. Pharmacol.* **22**: 250-257 (1982).

Nathanson, N. M.. Binding of agonists and antagonists to muscarinic acetylcholine receptors on intact cells. *J. Neurochem.* **41**: 1545-1549 (1983).

Nelson, D. L., A. Herbet, S. Bourgoin, J. Glowinski, and M. Hamon. Characteristics of central 5-HT receptors and their adaptive changes following intracerbral 5,7-dihydroxytryptamine administration in the rat. *Mol. Pharmacol.* **14**: 983-995 (1978).

Nelson, D. L., P. J. Monroe, G. Lambert and H. I. Yamamura. [^{3}H]Spiroxatrine labels a serotonin$_{1A}$-like site in the rat hippocampus. *Life Sci.* **41**: 1567-1576 (1987).

Neubig, R. H., R. D. Gantzos and R. S. Brasier. Agonist and antagonist binding to α_2-adrenergic receptors in purified membranes from human platelets. Implications of receptor-inhibitory nucleotide-binding protein stoichiometry. *Mol. Pharmacol.* **28**: 475-486 (1985).

Neubig, R. R., R. D. Gantzos and W. J. Thomsen. Mechanism of agonist and antagonist binding to alpha $_2$ adrenergic receptors: Evidence for a precoupled receptor-guanine nucleotide protein complex. *Biochemistry* **2**: 2374-2384 (1988).

Norman, A. B., G. Battaglia and I. Creese. [^{3}H]-WB-4101 labels the 5-HT_{1A} serotonin receptor subtype in rat brain. *Mol. Pharmacol.* **28**: 487-494 (1985).

Norman, A. B., M. T. Borchers, T. J. Wachendorf, A. L. Price and P. R. Sanberg. Loss of D_1 and D_2 dopamine receptors and muscarinic cholinergic receptors in rat brain following in vitro polytron homogenization. *Brain Res. Bull.* **22**: 633-636 (1989).

Ohara, K., K. Haga, G. Berstein, T. Haga, A. Ichiyama and K. Ohara. The interaction between D-2 dopamine receptors and GTP-binding proteins. *Mol. Pharmacol.* **33**: 290-296 (1988).

Olah, M. E., K. A. Jacobson and G. L. Stiles. Purification and characterization of bovine cerebral cortex A_1 adenosine receptor. *Arch. Biochem. Biophys.* **283**: 440-446 (1990).

Onodera, H. and K. Kogure. Differential localization of adenosine A_1 receptors in the rat hippocampus: quantitative autoradiographic study. *Brain Res.* **458**: 212-217 (1988).

Ott, S. and T. Costa. Guanine nucleotide-mediated inhibition of opioid agonist binding. Modulatory effects of ions and of receptor occupancy. *Biochem. Pharmaco.l* **38**: 1931-1939 (1989).

Palacios, J. M., A. Pazos, M. M. Dietl, M. Schlumpf and W. Lictensteiger. The ontogeny of brain neurotensin receptors studied by autoradiography. *Neuroscience* **25**: 307-317 (1988).

Palacios, J. M., A. Pazos and D. Hoyer. Characterization and mapping of $5\text{-}HT_{1A}$ sites in the brain of animals and men. *Brain 5-HT$_{1A}$ Receptors.* 67-81 (1987).

Palacios, J. M., A. Probst and R. Cortes. Mapping receptors in the human brain. *Trends Neurosci.* **9**: 284-289 (1986).

Parkinson, F. E. and B. B. Fredholm. Autoradiographic evidence for G-protein coupled A2-receptors in rat neostriatum using [^{3}H]-CGS 21680 as a ligand. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **342**: 85-89 (1990).

Paxinos, G. and C. Watson. The rat brain in stereotaxic coordinates. Academic Press, New York (1986).

Pazos, A., R. Cortes and J. M. Palacios. Quantitative autoradiographic mapping of serotonin receptors in the rat brain II. Serotonin-2 receptors. *Brain Res.* **346**: 231 (1985).

Pazos, ., D. Hoyer, M. M. Dietl and J. M. Palacios. Autoradiography of serotonin receptors. *Neuronal Serotonin.* 507-543 John Wiley, New York (1988).

Pazos, A. and J. M. Palacios. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. *Brain Res.* **346**: 205-220 (1985).

Pazos, A., A. Probst and J. M. Palacios. Serotonin receptors in the human brain-III. Autoradiographic mapping of serotonin-1 receptors. *Neuroscience* **21**: 97-122 (1987a).

Pazos, A., A. Probst and J. M. Palacios. Serotonin receptors in the human brain-IV. Autoradiographic mapping of serotonin-2 receptors. *Neuroscience* **21**: 123-140 (1987b).

Pedigo, N. W., H. I. Yamamura and D. L. Nelson. Discrimination of multiple [^{3}H]-5-HT binding sites by the neuroleptic spiperone in rat brain. *J. Neurochem.* **36**: 220-226 (1981).

Pelleg, A. and G. Burnstock. Physiological importance of ATP released from nerve terminals and its degradation to adenosine in humans. *Circulation* **82**: 2061-2067 (1990).

Phillis, J. W. and M. H. O'Regan. The role of adenosine in the central actions of the benzodiazepines. *Prog. Neuro-Psychopharmacol. Biol. Psychiat.* **12**: 389-404 (1988).

Radja, F., G. Daval, M. B. Emerit, M. C. Gallissot, M. Hamon and D. Vergé. Selective irreversible blockade of 5-hydroxytryptamine$_{1A}$ and 5-hydroxytryptamine$_{1C}$ receptor binding sites in the rat brain by 8-MeO-2'-chloro-PAT:. *Neuroscience* **31**: 723-733 (1989).

Rainbow, T. C., A. Biegon and D. J. Berck. Quantitative receptor autoradiography with tritium-labeled ligands: comparison of biochemical and densitometric measurements. *J. Neurosci. Meth.* **11**: 231-241 (1984a).

Rainbow, T. C., B. Parsons and B. B. Wolfe. Quantitative autoradiography of $beta_1$ and $beta_2$ receptors in rat brain. *Proc. Natl. Acad. Sci. USA* **81**: 1585-1589 (1984b).

Raymond, J. R., A. Fargin, J. P. Middleton, J. M. Graff, D. M. Haupt, M. G. Caron, R. J. Lefkowitz and V. W. Dennis. The human 5-HT_{1A} receptor expressed in HeLa cells stimulates sodium-dependent phosphate uptake via protein kinase C. *J. Biol. Chem.* **264**: 21943-219 (1989).

Richardson, P. J., S. J. Brown, E. M. Bailyes and J. P. Luzio. Ectoenzymes control adenosine modulation of immunoisolated cholinergic synapses. *Nature* **327**: 232-234 (1987).

Rydelek, F. L., M. Teitler, P. W. Fletcher, A. M. Ismaiel and R. A. Glennon. NAN-190: Agonist and antagonist interactions with brain 5-HT_{1A} receptors. *Brain Res.* **532**: 191-196 (1990).

Sabouni, M. H., M. V. Ramagopal and S. J. Mustafa. Relaxation by adenosine and its analogs of potassium-contracted human coronary arteries. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **341**: 388-390 (1990).

Sattin, A. and T. W. Rall. The effect of adenosine and adenine nucleotides on the cyclic adenosine 3', 5'-phosphate content of guinea pig cerebral cortex slices. *Mol. Pharmacol.* **6**: 13-23 (1970).

Schlegel, J. R. and S. J. Peroutka. Nucleotide interactions with 5-HT_{1A} binding sites directly labeled by [^{3}H]-8-hydroxy-2-(Di-n-propylamino) tetralin ([^{3}H]-8-OH-DPAT). *Biochem. Pharmacol.* **35**: 1943-1949 (1986).

Schoeffter, P. and D. Hoyer. 5-Hydroxytryptamine 5-HT_{1B} and 5-HT_{1D} receptors mediating inhibition of adenylate cyclase activity. Pharmacological comparison with special reference to the effects of yohimbine, rauwolscine and some beta-adrenoceptor antagonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**: 285-292 (1989).

Schwabe, U. and T. Trost. Characterization of adenosine receptors in rat brain by (-) [^{3}H]-N6-phenylisopropyladenosine. *Naunyn-Schmeideberg's Arch. Pharmacol.* **313**: 179-187 (1980).

Schwabe, U., D. Ukena and M. J. Lohse. Xanthine derivatives as antagonists at A_1 and A_2 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **330**: 212-221 (1985).

Shapiro, L. E. and T. R. Insel. Ontogeny of oxytocin receptors in rat forebrain: A quantitative study. *Synapse* **4**: 259-266 (1989).

Shearman, A. R. and P. G. Strange. Guanine nucleotide effects on agonist binding to serotonin $5HT_2$ receptors in rat frontal cortex. *Biochem. Pharmacol.* **37**: 3097 (1988).

Sijbesma, H., J. Schipper and E. R. De Kloet. The anti-aggressive drug eltoprazine preferentially binds to 5-HT_{1A} and 5-HT_{1B} receptor subtypes in rat brain: Sensitivity to guanine nucleotides. *Eur. J Pharmacol.* **187**: 209-223 (1990).

Snowhill, E. W. and M. Williams. [^{3}H]-Cyclohexyladenosine binding in rat brain: A pharmacological analysis using quantitative autoradiography. *Neurosci. Lett.* **68**: 41-46 (1986).

Snyder, S. H.. Adenosine as a neuromodulator. *Ann. Rev. Neurosci.* **8**: 103-124 (1985).

Stadel, J. M., A. DeLean and R. J. Lefkowitz. A high affinity agonist-Beta-adrenergic receptor complex is an intermediate for catecholamine stimulation of adenylate cyclase in turkey and frog erythrocyte membranes. *J. Biol. Chem.* **255**: 1436-1441 (1980).

Stiles, G. L.. A_1 adenosine receptor-G protein coupling in bovine brain membranes: Effects of guanine nucleotides, salt and solubilization. *J. Neurochem.* **51**: 1592-1598 (1988).

Stone, G. A., M. F. Jarvis, M. A. Sills, B. Weeks, E. B. Snowhill and M. Williams. Species differences in high affinity adenosine A_2 binding sites in striatal membranes from mammalian brain. *Drug Dev. Res.* **15**: 31-46 (1988).

Strange, P. G.. The structure and mechanism of neurotransmitter receptors. Implications for the structure and function of the central nervous system. *Biochem. J.* **249**: 309-318 (1988).

Strange, P. G.. States and subtypes of the 5-HT_2 serotonin receptor: Interpretation of the data. *J. Neurochem.* **54**: 1085 (1990).

Stratford, C. A., G. L. Tan, M. W. Hamblin and R. D. Ciaranello. Differential inactivation and G-protein reconstitution of subtypes of [^{3}H]5-hydroxytryptamine binding sites in brain. *Mol. Pharmacol.* **34**: 527-536 (1988).

Ströher, M., C. Nanoff and W. Schütz. Differences in the GTP-regulation of membrane-bound and solubilized A_1-adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**: 87-92 (1989).

Stryer, L. G. G-proteins: a family of signal transducers. *Ann. Rev. Cell Biol.* **2**: 391-419 (1986).

Taylor, C. W.. The role of G-proteins in transmembrane signalling. *Biochem. J.* **272**: 1-13 (1990a).

Taylor, D. P.. Serotonin agents in anxiety. *Ann. N. Y. Acad. Sci.* **600**: 545-557 (1990b).

Teitler, M., S. Leonhardt, E. L. Weisberg and B. J. Hoffman. 4-[^{125}I]Iodo-(2,5-dimethoxy) phenylisopropylamine and [^{3}H]ketanserin labeling of 5-hydroxytryptamine$_2$ ($5HT_2$) receptors in mammalian cells transfected with a rat $5HT_2$ cDNA: Evidence for multiple states and not multiple $5HT_2$ receptor subtypes. *Mol. Pharmacol.* **38**: 594-598 (1990).

Thor, K. B., S. A. Blitz and C. J. Helke. Discrete localization of high-density 5-HT_{1A} binding sites in the midline raphe and parapyramidal region of the ventral medulla oblongata of the rat. *Neurosci. Lett.* **108**: 249-254 (1990).

Titeler, M., R. A. Lyon and R. A. Glennon. Radioligand binding evidence implicates the brain 5-HT_2 receptor as a site of action for LSD and phenylisopropylamine hallucinogens. *Psychopharmacology* **94**: 213-216 (1988).

Torda, T. and J. M. Saavedra. Determination of guanine nucleotide sensitivity of [^{125}I]-neuropeptide Y binding in the rat pituitary gland by quantitative autoradiography. *Neuroendocrinology* **52**: 361-367 (1990).

Trussell, L. O. and M. B. Jackson. Dependence of an adenosine-activated potassium current on a GTP-binding protein in mammalian central neurons. *J. Neurosci.* 7: 3306-3316 (1987).

Ukena, D., E. Poeschla, and U. Schwabe. Guanine nucleotide and cation regulation of radioligand binding to R*i* adenosine receptors of rat fat cells. *Naunyn-Schmiedebergs's Arch. Pharmacol.* 326: 241-247 (1984).

Van Calker, D., M. Muller and B. Hamprecht. Adenosine regulates via two different types of receptors the accumulation of cAMP in cultured brain cells. *J. Neurochem.* 33: 990-1005 (1979).

Waeber, C., P. Schoeffteer, J. M. Palacios and D. Hoyer. Molecular pharmacology of 5-HT_{1D} recognition sites: Radioligand binding studies in human pig and calf brain membranes. *Naunyn-Schmiedebergs's Arch. Pharmacol.*. 337: 595-601 (1988).

Waeber, C., P. Schoeffter, D. Hoyer and J. M. Palacios. The serotonin 5-HT_{1D} receptor: A progress review. *Neurochem. Res.* 15: 567-582 (1990).

Waeber, C., P. Schoeffter, J. M. Palacios and D. Hoyer. 5-HT_{1D} receptors in guinea-pig and pigeon brain. Radioligand binding and biochemical studies. *Naunyn-Schmiedebergs's Arch. Pharmacol.* 340: 479-485 (1989).

Weber, R. G., C. R. Jones, M. J. Lohse and J. M. Palacios. Autoradiographic visualization of A_1 adenosine receptors in rat brain with [^{3}H]8-cyclopentyl-1,3-dipropylxanthine. *J. Neurochem.* 54: 1344-1353 (1990).

Welner, S. A., M. C. De, J. Desroches, P. Desjardins and C. B. Suranyi. Autoradiographic quantification of serotonin$_{1A}$ receptors in rat brain following antidepressant drug treatment. *Synapse* 4: 347-352 (1989).

Werling, L. L., P. S. Puttfarcken and B. M. Cox. Multiple agonist-affinity states of opioid receptors: Regulation of binding by guanyl nucleotides in guinea pig cortical NG108-15, and 7315c cell membranes. *Mol. Pharmacol.* 33: 423-431 (1988).

Yeung, S. M. H. and R. D. Green. Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5'-guanylylimidodiphosphate. *J. Biol. Chem.* **258**: 2334-2339 (1983).

Yeung, . M. H. and R. H. Green. [^{3}H]5'-N-ethylcarboxamide adenosine binds to both Ra and Ri adenosine receptors in rat striatum. *Naunyn-Schmeideberg's Arch.Pharmacol.* **325**: 218-225 (1984).

Yocca, F. D., D. K. Hyslop, D. W. Smith and S. Maayani. BMY-7378, a buspirone analog with high affinity, selectivity and low intrinsic activity at the 5-HT_{1A} receptor in rat and guinea pig hippocampal membranes. *Eur. J. Pharmacol.* **137**: 293-294 (1987).

Zahniser, N. R. and P. B. Molinoff. Effect of guanine nucleotides on striatal dopamine receptors. *Nature* **275**: 453-455 (1978).

Zarbin, M., J. M. Palacios, J. K. Wamsley and M. J. Kuhar. Axonal transport of beta-adrenergic receptors. Antero- and retrogradely transported receptors differ in agonist affinity and nucleotide sensitivity. *Mol. Pharmacol.* **24**: 341-348 (1983).

Zarbin, M. A., J. Baraban and P. Worley. Autoradiographic distribution of forskolin and phorbol ester binding sites in the retina. *Brain Res.* **497**: 334-343 (1989).

Zarbin, M. A., J. K. Wamsley and M. J. Kuhar. Anterograde transport of opioid receptors in rat vagus nerves and dorsal roots of spinal nerves: Pharmacology and sensitivity to sodium and guanine nucleotides. *Exp. Brain. Res.* **81**: 267-278 (1990).

Zgombick, J. M., S. G. Beck, C. D. Mahle, R. B. Craddock and S. Maayani. Pertussis toxin-sensitive guanine nucleotide-binding protein(s) couple adenosine A_1 and 5-hydroxytryptamine$_{1A}$ receptors to the same effector systems in rat hippocampus: Biochemical and electrophysiological studies. *Mol. Pharmacol.* **35**: 484-494 (1989).