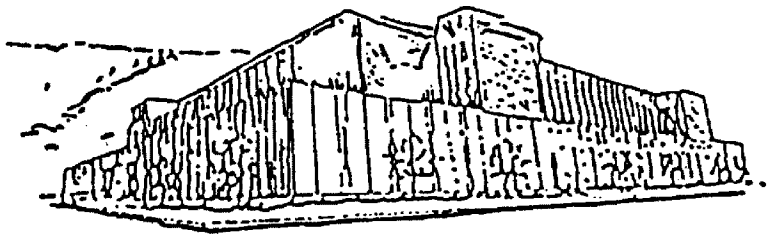
1995

Characteristics of the 5-HTb1A receptor in rabbit brain

John T. Weber
The University of Montana



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CHARACTERISTICS OF THE 5-HT_{1A} RECEPTOR IN RABBIT BRAIN

by

John T.Weber

B.S. Eastern Michigan University, 1992

presented in partial fulfillment of the requirements

for the degree of

Master of Science

The University of Montana

1995

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

July 14, 1995

Date

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Weber, John T., M.S., July 1995 Pharmacy

Characteristics of the 5-HT_{1A} Receptor in Rabbit Brain (69pp.)

Director: Keith K. Parker, Ph.D.

Serotonin exerts its effects through a group of receptors in the central nervous system. Among these, 5-HT_{1A} remains one of the most studied of the serotonin receptors, and is implicated in several neuropathologies. Although the 5-HT_{1A} receptor has been extensively characterized in several mammalian species, it has yet to be characterized in the rabbit. This study was undertaken to characterize the 5-HT_{1A} receptor in rabbit cerebral cortex in terms of ligand binding and G protein linkage. The 5-HT_{1A} binding site in rabbit cerebral cortex exhibits similar characteristics to 5-HT_{1A} receptors found in other mammalian species, including the rat and human. Based on the results, the physiology and pathology of the 5-HT_{1A} receptor can be studied confidently in the rabbit brain.

Several techniques used to investigate receptor-ligand interactions require purified receptor. Therefore, studies were conducted to investigate if cloned human 5-HT_{1A} receptor expressed in Chinese Hamster Ovary (CHO) cells maintains the characteristics of membrane bound receptor after solubilization. Solubilized human 5-HT_{1A} receptor appears to exhibit similar characteristics in comparison to membrane bound receptor.

Parthenolide is believed to be the active constituent in feverfew, a plant that has been used as a folklore remedy for migraine. This substance has yet to be screened at the serotonin receptors which have been implicated in migraine pharmacotherapy (5-HT_{1A}, 5-HT_{1D} and 5-HT_2). Therefore, the interactions of this substance at these receptors was investigated. Parthenolide showed no activity at the 5-HT_{1A} and 5-HT_{1D} receptors. Binding was exhibited at 5-HT_2 receptors, but with low affinity. Parthenolide does not appear to exhibit its reputed effects by binding directly to serotonin receptors in the brain. It may produce its effects by another mechanism. Also, another constituent in the feverfew plant, or parthenolide in combination with other consituents, may be responsible for the plants anti-migraine effects.

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ACKNOWLEDGEMENTS

I would like extend a special thank you to my major advisor, Dr. Keith Parker. Your guidance in the lab and attitudes towards both science, and life in general, I will always cherish. Thanks for putting up with my odd hours of work, and for instilling in me the confidence I did not have when I first arrived at the University of Montana. Thanks to the School of Pharmacy and the Department of Pharmaceutical Sciences for enabling me to come to Montana to carry on my education. To Dr.'s Chuck Eyer, Craig Johnston, and Walt Hill for sitting on my thesis committee and for their advice. To Dr.'s Bryan Tarr, Jerry Smith, Mike Minnick, Vernon Grund and John Raymond for either supplying, or helping to supply, materials for this project. To Marta Morlan, Mary-Frances O'Connor and Susan Wilson, for their technical assistance in the laboratory. Thanks Mom, Dad, Keith, Brian, and Joshua. For making growing up fun, and instilling in me the morals which have made me the person that I am today. To my fellow students, who have made the School of Pharmacy an enjoyable atmosphere in which to study. Lastly, thanks to Missoula and Montana. For your beautiful mountains and rivers. For your endless hiking trails. For the friendliness of your people, which means more to me than anything else in the world , and has made my two years here the most enjoyable I have yet to experience. I will miss the friendships that I have developed here and can only hope that someday I will return.

INTRODUCTION

I. SEROTONIN

Since the mid-nineteenth century, researchers had been aware of a substance found in serum that caused contractions of smooth muscle organs. In 1948, Rapport, Green, and Page at the Cleveland Clinic, succeeded in isolating and characterizing this serum tonic factor that was released from platelets during clotting of blood (Rapport, et.al., 1948). They appropriately named this substance "serotonin". Meanwhile, in Italy, Vittorio Erspamer was characterizing a substance that was found in high concentrations in the chromaffin cells of the intestinal mucosa (as reviewed by Erspamer, 1966). This substance also caused contraction of smooth muscles, and it was named "enteramine". Both substances were eventually purified and crystallized and in 1952, were shown to be the same chemical, 5-hydroxytryptamine (5-HT). After the isolation and synthesis of serotonin, interest was gained in examining other tissues of the body to see if this substance was present in structures other than blood platelets and the intestine. This led to the discovery by Twarog and Page in 1953 that serotonin was found in relatively large amounts in the brain (Twarog and Page,1953).

With the discovery of 5-HT in the central nervous system, theories arose that various forms of mental illness could be attributed to abnormalities in its synthesis. This awareness was heightened by the observation that the tranquilizing substance, reserpine, depleted 5-HT (Pletscher,et.al.,1955), and that throughout the depletion a distinct behavioral depression

was observed. Further strengthening these beliefs was the observation that the potent hallucinogenic substance, lysergic acid diethylamide (LSD), resembled 5-HT structurally, and acted as a 5-HT antagonist on smooth muscle preparations (Gaddum and Hameed,1954).

These beliefs held true and 5-HT is arguably the neurotransmitter which is most intricately involved with modern neuropsychopharmacology. 5-HT has been found to play an important, but not an exclusive, role in several neuropathological syndromes. The correlation between behavioral depression and decreased 5-HT activity has been established. The fact that tricyclic antidepressants and other novel antidepressants inhibit the re-uptake of 5-HT adds support to this hypothesis (Peroutka, et.al., 1989;van Zweiten,et.al.1990). There is evidence that 5-HT plays a significant role in changes in core behaviors that are associated with depression. These behaviors include sleep, appetite, body temperature, motor activity, cognitive functions and sexual behavior (Meltzer, 1990). Abnormalities in 5-HT are also implicated in anxiety (Carli, et al., 1989; Peroutka, et.al., 1989) and panic disorder (Lesch et.al., 1992). 5-HT has also been shown to play an important role in the pathology of migraine headache. Several pharmaceutical agents have been used in treating this latter ailment. All anti-migraine drugs, with the exception of aspirin and related compounds, appear to have important direct, or indirect, effects on the 5-HT receptor system (Peroutka, 1990b; Raskin, 1990).

II. 5-HT RECEPTORS

In 1957, Gaddum and Picarelli demonstrated that there were two types of receptors for 5-HT in the guinea-pig ileum (Gaddum and Picarelli, 1957). They classified these receptors as M receptors, which could be blocked with morphine, and D receptors, which could be blocked with dibenzyline. A little over two decades later, Peroutka and Snyder demonstrated that [^{3}H]Lysergic acid diethylamide (LSD) labeled two distinct binding sites in rat frontal cortex (Peroutka and Snyder, 1979). They classified these receptors as 5-HT_1 and 5-HT_2. 5-HT_1 receptors had high affinity for 5-HT itself and corresponded to sites labeled by [^{3}H]5-HT. The 5-HT_2 receptors corresponded to sites labeled by [^{3}H]spiperone and had a much lower affinity for 5-HT. It was later found that the 5-HT_1 and 5-HT_2 receptors subtypes did not correspond to the M and D receptors that were defined in the guinea-pig ileum. Subsequently, the 5-HT_3 receptor was described in the periphery, and potent, radiolabeled 5-HT_3 antagonists were developed using peripheral tissue models (Brittain et.al., 1987; Fozard, 1984; Richardson et.al., 1985). The 5-HT_3 receptor was then described in the brain (Kilpatrick, et al., 1987), demonstrating that there were more than just two types of receptors for 5-HT in this tissue.

These findings ushered in the modern era of the study of 5-HT receptors in the central nervous system. During the past couple of decades, several subtypes or "families" of 5-HT receptors have been identified on the basis of molecular biological, pharmacological and biochemical properties (Peroutka, et.al.,1990). Now, much with the aid of molecular biology (Julius, 1991), seven major subtypes of receptors for 5-HT are understood and sor

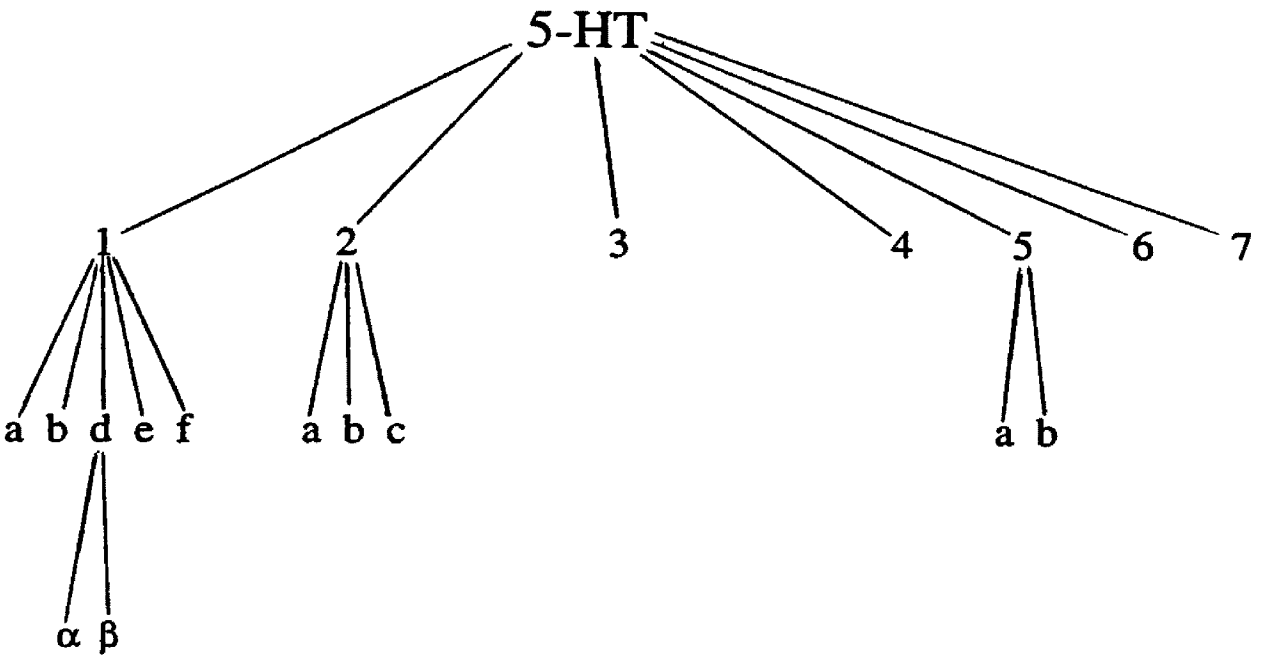

Fig. 1. Schematic Representation of the Current Classification of 5-HT Receptors.

of these subtypes are further divided (Hoyer, et.al., 1994; Zifa and Fillion, 1992). Figure 1 represents a schematic breakdown of the current classification of the 5-HT receptors.

5-HT receptors are not only divided by molecular genetics and binding specificities, but also by signal transduction processes which link binding events to effector consequences (Hoyer, et.al., 1994, Zifa and Fillion, 1992). Figure 2 represents an example of a membrane bound receptor coupled to an effector system. With the probable exception of the 5-HT_3 receptor, which is an ion channel (Maricq, et.al., 1991), the remaining 5-HT receptor subtypes are members of the superfamily of seven transmembrane (Figure 3), G protein coupled receptors (Strader, et.al., 1994).

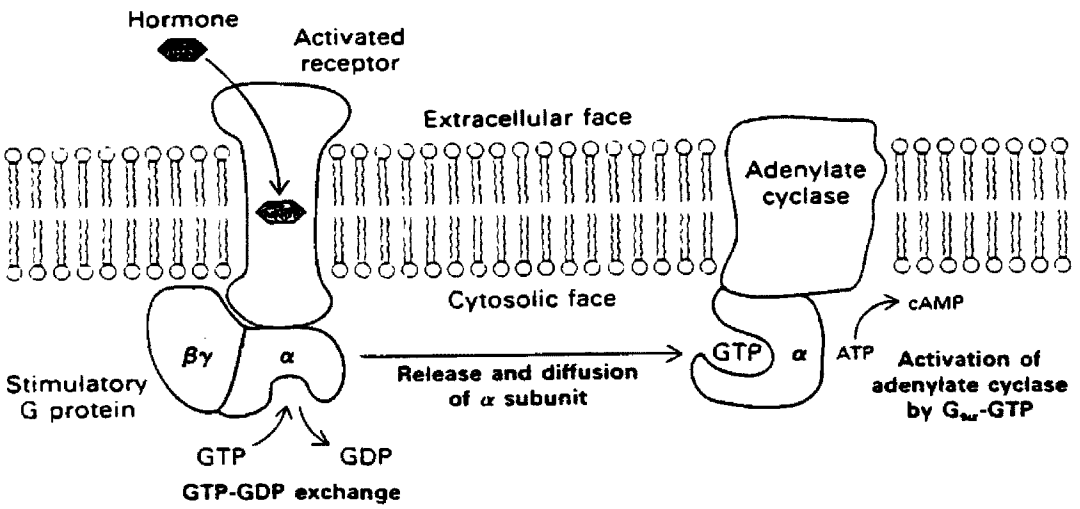

Fig. 2. Membrane bound receptor is activated by hormone (or drug) which initiates effector consequences eventually leading to a physiological response. This diagram shows an example of a receptor which is coupled to adenylate cyclase and leads to an increase in cAMP formation. (from Stryer, 1988).

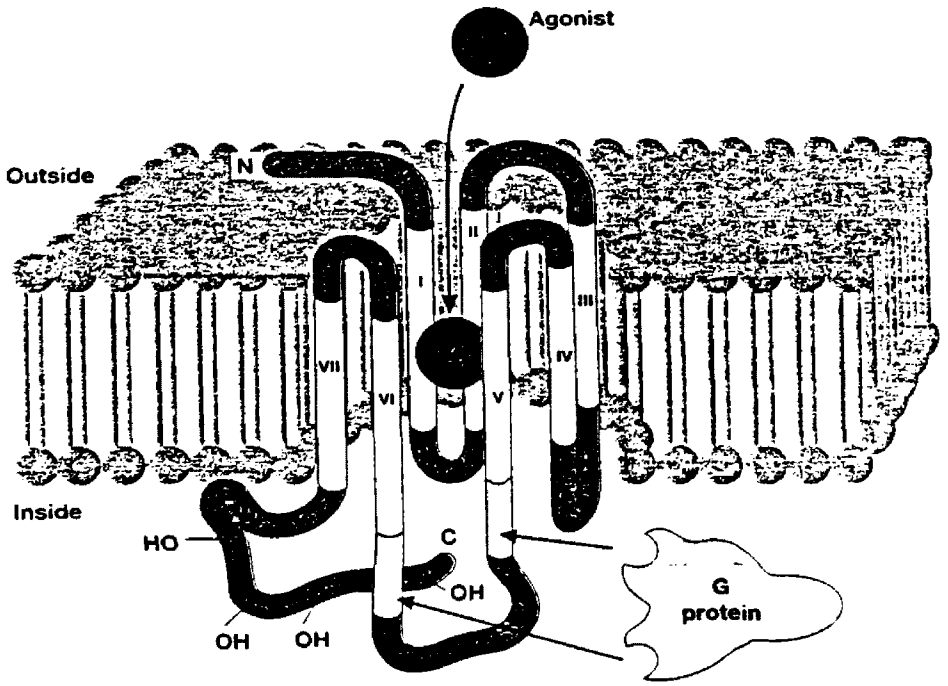

Fig.3. Representation of the structure of a 7 transmembrane receptor. All 5-HT receptors, with the exception of 5-HT_3, are considered members of this superfamily of G protein coupled receptors. (from Katzung, 1995).

Guanine nucleotide binding regulatory proteins (G proteins) are proteins which bind to Guanosine triphosphate (GTP), thus transmitting receptor binding signals to effectors and second messenger systems. Figure 4 represents signal transduction through G protein systems. Although different effector systems have been described for 5-HT_1 receptors (Hoyer, et al., 1994; Raymond, et al., 1993), they have generally been described as being negatively coupled to adenylate cyclase and therefore, decrease cAMP formation. 5-HT_2 receptors have been generally thought to be positively coupled to phospholipase C, thus leading to an increase in inositol triphosphate production. 5-HT_4, 5-HT_6, and 5-HT_7 receptors have been found to be positively coupled to adenylate cyclase and lead to an increase in cAMP production. The effector and second messenger systems associated with the 5-HT_5 receptor are not currently understood. Table 1 lists all of the currently known 5-HT receptors and the effector and second messenger systems that are associated with them, as well as radiolabeled ligands currently used to identify the receptors. Regardless of the effector and second messenger systems involved, G proteins mediate receptor coupling (Hoyer, et al., 1994).

III. 5-HT_{1A} RECEPTORS

A. Identity

The early 1980's saw the further subdivision of the 5-HT_1 receptor (Fozard, 1987). Of these, 5-HT_{1A} represents one of the best studied of the 5-HT receptors (Fargin, et al.,

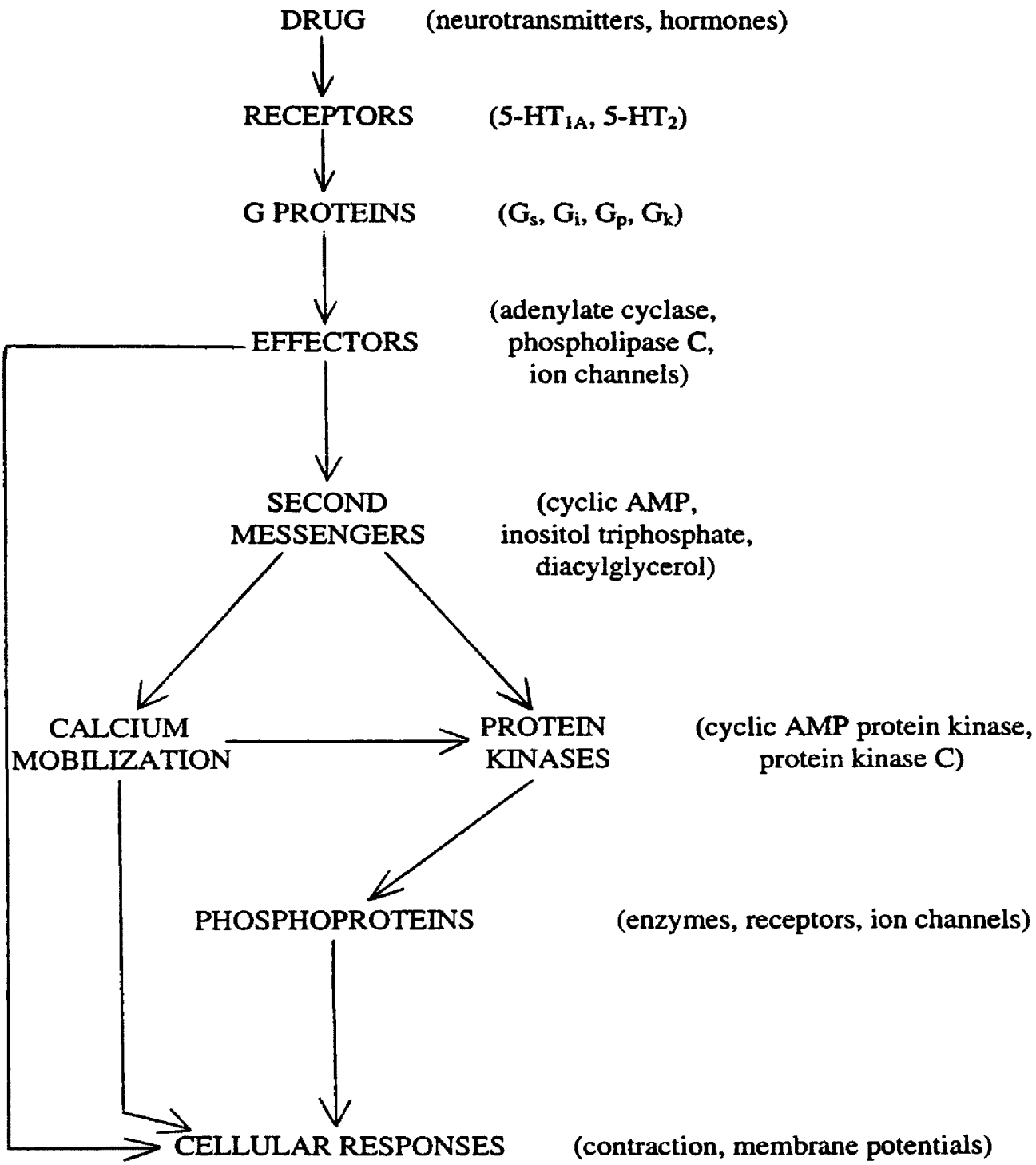

Fig 4. Signal Transduction through G protein systems. Examples at each step are indicated in parentheses. G proteins that stimulate adenylate cyclase (G_s), inhibit adenylate cyclase (G_i), activate potassium channels (G_k), and that stimulate phopholipase C (G_p) are listed as examples. (from DeVivo and Maayani, 1989).

Serotonin Receptor	Radiolabeled Ligands	Initial Result of Receptor Activation	Second Messenger Affected
5-HT_{1A}	[^{3}H]8-OH-DPAT	Inhibition of adenylate cyclase	↓ Cyclic AMP
5-HT_{1B}	[^{125}I]GTI, [^{3}H]5-HT		
5-$HT_{1D\alpha}$ 5-$HT_{1D\beta}$	[^{125}I]GTI, [^{3}H]5-CT		
5-HT_{1E}	[^{3}H]5-HT		
5-HT_{1F}	[^{125}I]LSD		
5-HT_{2A}	[^{3}H]ketanserin, [^{3}H]spiperone	Phospholipase C activation	Diacylglycerol and IP_3
5-HT_{2B}	[^{3}H]5-HT, [^{125}I]DOI		
5-HT_{2C}	[^{3}H]mesulergine		
5-HT_3	[^{3}H]zacopride	Opening of ion channel	Calcium
5-HT_4	[^{3}H]GR113808, [^{3}H]SB207710	Stimulation of adenylate cyclase	↑ Cyclic AMP
5-HT_{5A}	[^{3}H]5-HT,[^{125}I]LSD	???	???
5-HT_{5B}	[^{3}H]5-HT,[^{125}I]LSD		
5-HT_6	[^{3}H]5-HT,[^{125}I]LSD	Stimulation of adenylate cyclase	↑ Cyclic AMP
5-HT_7	[^{3}H]5-HT,[^{125}I]LSD	Stimulation of adenylate cyclase	↑ Cyclic AMP

TABLE 1. CLASSIFICATION OF 5-HT RECEPTORS. * It should be noted that the 5-HT_{1E}, 5-HT_{1F}, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6 and 5-HT_7 receptors were characterized predominately by molecular biological criteria. Also, [^{3}H]5-HT labels all 5-HT_1 sites and can only be used in combination with adequate masking ligands.

1988; Hoyer, et al., 1994). The initial heterogeniety of 5-HT_1 receptors was described relative to the drug spiperone (Schnellman, et al., 1984). 5-HT_{1A} sites have relatively high affinity for spiperone, while 5-HT_{1B} sites show a much lower affinity. These two types of [^{3}H]5-HT binding sites were originally defined on the basis of their affinities for unlabeled spiperone, and should not be confused with the 5-HT_2 site, which is defined by high affinity binding of [^{3}H]spiperone or [^{3}H]ketanserin. Table 1 lists radioligands that are now used to characterize these receptors, as well as other 5-HT receptors.

5-HT_{1A} sites are further defined by their high affinity for aminotetralins, of which 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) is the best known. Additionally, 5-HT_{1A} sites show high affinity for 5-carboxamidotryptamine (5-CT), 5-methoxydimethyltryptamine, ipsapirone, and buspirone (Peroutka, 1988). The pharmacological typing of this site has been further strengthened by the molecular cloning of a receptor from rat (Albert, et al., 1990) and human (Kobilka, et al., 1987; Fargin, et al., 1988). When this cloned receptor is expressed in cell cultures, it demonstrates the pharmacological profile as described above, and lacks specific binding characteristics of other 5-HT_1 receptors. These binding characteristics, in association with properties of tissue localization and signal transduction, leads to the conclusion that a unique 5-HT_{1A} receptor is present physiologically (Peroutka, 1988).

B. Clinical Implications

From a clinical perspective, the 5-HT_{1A} receptor is specifically implicated in several neuropathological phenomena, and therefore represents a potential target for pharmaceutical intervention.

The finding that buspirone is a non-benzodiazepine anxiolytic drug (Goa and Ward, 1986) and the fact that this drug has a high affinity for the 5-HT_{1A} receptor (Traber and Glaser, 1987) stimulated interest in the involvement of this receptor with anxiety. Clinical development of selective 5-HT_{1A} agents as potential anxiolytics is currently ongoing (Ablinsson, et al., 1994, Peroutka. et al., 1989). 5-HT_{1A} receptor dysfunction has been linked to the pathophysiology of panic disorder as well. 5-HT_{1A} agonists, such as ipsapirone, have shown considerable potential in treating this disorder (Lesch, et al., 1992).

The 5-HT_{1A} receptor has also been implicated in behavioral depression. 8-OH-DPAT, a highly selective agonist at 5-HT_{1A} receptors, has demonstrated anti-depressant activity in certain animal models of depression (Cervo and Samain, 1987; Kennett, et al., 1987). Further, the novel anti-depressant, cericlamine, has been shown to desensitize presynaptic 5-HT_{1A} autoreceptors (Jolas, et al., 1994). This leads to an increase in 5-HT neurotransmission, an analogous mechanism to that of tricyclic antidepressants, which block the reuptake of 5-HT, thus increasing the amount of 5-HT at neuronal synapses.

The 5-HT_{1A} receptor has also gained interest in its possible involvement in migraine headache. Dihydroergotamine, which has long been used for the treatment of acute migraine attacks, has been shown to be a potent agonist at 5-HT_{1A} receptors (McCarthy and Peroutka, 1989).

C. Anatomical Localization

The 5-HT_{1A} receptor has been identified in several mammalian species (Schnellman, et al., 1984). The receptor has been found in high densities in the limbic system of mammalian species that have been investigated, including the rat and human (Palacios et al., 1990). It apparently exists as a post-synaptic receptor in these areas of the brain. It has also been found in high densities in the raphe nuclei of mammals, where it exists as a pre-synaptic autoreceptor (Palacios, et al., 1990).

D. Rabbit Brain 5-HT_{1A} Receptor

Although the 5-HT_{1A} receptor has been identified in the brain of the rabbit (Xiong and Nelson, 1989), it has never been fully characterized in terms of ligand binding and G protein linkage. Pre-synaptic autoreceptors in the rabbit brain are apparently not of the 5-HT_{1A} type (Limberger, et al., 1991; Waeber and Palacios, 1992). The type of receptor that was identified by Xiong and Nelson (1989) is the post-synaptic 5-HT_{1A} receptor. The rabbit is used extensively in biomedical research, especially in cardiovascular and pharmacological studies (Benedini, et al., 1995.; Ferrari, et al., 1991; Leff and Martin, 1988; Woodley and Barclay, 1994). In particular, the rabbit is often used when investigating 5-HT receptors in the brain (Dewar, et al., 1990; Feuerstein, et al., 1987; Schoups, et al., 1986; Schoups and DePotter, 1988).

The complete characterization of the 5-HT_{1A} receptor in rabbit brain will allow for an even greater understanding of the receptor across mammalian species. There is a high

amount of applicability to humans when similar results are seen across different species, especially those from various orders. Therefore, the study of 5-HT_{1A} receptor physiology and pathology could be confidently studied in the rabbit brain.

E. 5-HT_{1A} Characterization

A number of drugs have been shown to be useful in the characterization of 5-HT_{1A} receptors. A brief description of some of these drugs and their utility in this study follows:

8-OH-DPAT The aminotetralin, 8-OH-DPAT, was discovered in the early 1980's (Zifa and Fillion, 1992) and its discovery was soon followed by the development of the tritiated compound (Gozlan et al., 1983). It was found to be a highly selective, full agonist at the 5-HT_{1A} receptor, allowing for more detailed study of this specific receptor subtype. Other radioligands have been described for 5-HT_{1A}, but none have been able to surpass [^{3}H]8-OH-DPAT in its overall usefulness (Hoyer, et al., 1994).

Buspirone As previously described, the fact that buspirone is a non-benzodiazepine anxiolytic drug (Goa and Ward, 1986), and the finding that it has high affinity for the 5-HT_{1A} receptor (Traber and Glaser, 1987), sparked interest in the involvement of this receptor with anxiety. Buspirone is described as a partial agonist at 5-HT_{1A} receptors, meaning that it fully occupies these receptors, but produces less than the maximal response

produced by a full agonist. It is often used in displacement studies of [^{3}H]8-OH-DPAT when studying this receptor.

Propranolol Propranolol has been found to directly displace [^{3}H]5-HT from its binding sites in rat cortex (Middlemiss, 1984) providing evidence for an interaction of this beta-adrenoreceptor antagonist with 5-HT receptors. The (-) stereoisomer of propranolol was later found to block selective 5-HT_{1A} agonists (Sprouse and Aghajanian, 1986) and has therefore become useful in the characterization of this receptor as a 5-HT_{1A} antagonist.

Spiroxatrine The compound [^{3}H]spiroxatrine was initially described as labeling 5-HT_{1A} sites in the rat hippocampus (Nelson, et al., 1987). It is a selective agonist at the 5-HT_{1A} receptor and continues to be utilized by researchers in the study of this receptor (Fargin et al., 1988; Hoyer et al., 1994).

WB 4101 [^{3}H]2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane ([^{3}H]WB 4101), like spiroxatrine, was originally described as labeling 5-HT_{1A} sites in the rat brain (Norman et al., 1985). It is a selective agonist at these receptors as well, and its use continues for those investigating the 5-HT_{1A} receptor in the brain (Nelson, et al., 1987; Zifa and Fillion, 1992).

Methiothepin The compound methiothepin has a high affinity for 5-HT_{1A} receptors. Methiothepin displays antagonist activity at 5-HT_{1A} receptors, and it is often used in addition to other ligands when characterizing this receptor (Gozlan, et al., 1983; Hoyer, et al., 1985; Zifa and Fillion, 1992).

Dopamine The naturally occuring monoamine, dopamine, has low affinity for 5-HT_{1A} and 5-HT receptors as a whole. However, it is often used when characterizing the 5-HT_{1A} receptor, mainly as a control and for comparative purposes (Fargin, et al., 1988; Nelson, et al., 1987; Norman, et al., 1985).

Gamma-S-GTP and Gamma-S-ATP Stable Guanosine triphosphate (GTP) analogues are often used to determine if a receptor is G protein-linked. Guanine nucleotides decrease high affinity agonist binding to receptors by converting them to a low affinity state (Fargin, et al., 1988). Therefore, GTP should decrease the binding of agonists, like 8-OH-DPAT, for 5-HT_{1A} receptors. Adenosine triphosphate (ATP) analogues should not decrease high affinity binding, and are therefore used as a control when investigating G protein linkage of a receptor.

IV. HUMAN 5-HT$_{1A}$ RECEPTOR

The 5-HT$_{1A}$ receptor has been cloned from the human (Kobilka, et al., 1987, Fargin, et al., 1988), and this intronless gene encodes a protein which has been expressed in several tissue culture cell lines. The particular cell line in which the receptor is expressed can have a major bearing on the nature of signal transduction processes. Originally expressed in COS 7, a monkey kidney cell line, the receptor is negatively coupled to adenylate cyclas (Fargin, et al., 1989). When it is expressed in HeLa cells, it has been found to be negatively coupled to adenylate cyclase (Fargin, et al., 1989), but also positively coupled to protein kinase C (Raymond, et al., 1989). In fibroblast L cells, the receptor is positively coupled to phospholipase C (Liu, et al., 1991). When the receptor has been expressed in Chinese Hamster Ovary (CHO) cells, it has been found to be negatively coupled to adenylate cyclase (Raymond, et al., 1993), and this is the result which is most often reported from non-transfected systems in other species (Hoyer, et al., 1994; Zifa and Fillion, 1992).

Because the human 5-HT$_{1A}$ receptor expressed in CHO cells shows similar characteristics to the receptor found in non-transfected systems in other species, it should serve as a valid model of comparison to the characteristics of the rabbit brain 5-HT$_{1A}$ receptor.

Some techniques that are used to create a greater understanding of the interactions between receptors and ligands, such as multidimensional NMR (Dratz, et al., 1993), require purified receptor. The goal of solubilizing 5-HT receptors is to obtain a preparation that retains the pharmacological and biochemical characteristics of the membrane-bound

receptor. Binding studies and GTP inhibition studies with 5-HT_{1A} receptor solubilized from CHO cells can help to achieve this goal.

V. 5-HT INVOLVEMENT IN MIGRAINE

The early 1980's saw the beginning of thoughts that 5-HT receptors may be involved in the pathology of migraine headache (Houston and Vanhoutte, 1986; Janssen, 1983). With the exception of aspirin and other non-steroidal anti-inflammatory drugs that have been used in treating this ailment, all anti-migraine drugs appear to have either a direct or indirect effect on the 5-HT receptor system (Peroutka, 1990b; Raskin, 1990). Ergot alkaloids are the class of compounds that have been used most often in treating acute migraine attacks. One of these substances, dihydroergotamine, is most potent as a 5-HT_{1A} agonist, but also shows high affinity for the 5-HT_{1D} receptor (McCarthy and Peroutka, 1989). Sumatriptan, which is a novel acute migraine agent, is most potent at 5-HT_{1D} receptors as an agonist, but also displays high affinity for 5-HT_{1A} (McCarthy and Peroutka; 1989). Therefore, agonist activity at 5-HT_{1A} and/or 5-HT_{1D} receptors appears to be efficacious for treating acute migraine attacks. Some other useful anti-migraine substances are drugs that act as 5-HT_2 antagonists. An example in this class of compounds is methysergide, and these types of substances have been found to be most useful in migraine prophylaxis (Peroutka, 1990b).

VI. PARTHENOLIDE

A. Background

European feverfew, *Tanacetum parthenium*, is a plant that has been used as a folk remedy for migraine for several years (Awang, et al., 1991; Marles, et al., 1992). Parthenolide, one of the major compounds found in feverfew, is believed to be the active constituent of the plant (Awang, et al., 1991). The finding that parthenolide inhibits the release of 5-HT from blood platelets (Groenewegen and Hepinstall, 1990; Marles, et al., 1992) has stimulated interest in the interactions of this substance with the serotonergic system. This compound has yet to be investigated at the 5-HT receptors that have been implicated in migraine therapy (5-HT_{1A}, 5-HT_{1D} and 5-HT_2). Analysis of the interaction of parthenolide with these 5-HT receptors will help lead to a greater understanding of the mechanism of action behind this substance.

B. Screening at 5-HT_{1A}, 5-HT_{1D} and 5-HT_2 Receptors

After the characterization of the 5-HT_{1A} receptor in rabbit brain, parthenolide can be confidently screened at this receptor using the rabbit model. The 5-HT_{1D} (Limberger, et al., 1991) and 5-HT_2 (Schoups, et al., 1986) receptors have both been characterized in the rabbit brain, and therefore the interaction of parthenolide with these receptors can be legitimately studied in this species.

Drugs that have been used to study 5-HT_{1A} receptors have been previously described. The drugs that will be used to investigate the interaction of parthenolide with 5-HT_{1D} and 5-HT_2 receptors in this study are briefly described below:

5-CT [^{3}H]5-CT has been used as a selective ligand for investigating the 5-HT_{1D} receptor (Peroutka, 1988). However, the compound has also exhibited a high affinity for 5-HT_{1A} receptors. Therefore, when 5-CT is used, it is necessary to mask the 5-HT_{1A} receptors with a high amount of the 5-HT_{1A} selective ligand, 8-OH-DPAT.

Ketanserin [^{3}H]Ketanserin was the first potent 5-HT_2 antagonist identified (Leysen, et al., 1982) and although it binds with high affinity to all 5-HT_2 receptors, it has recently been shown to bind most specifically to 5-HT_{2A} receptors (Hoyer, et al., 1994). It has also been shown that [^{3}H]ketanserin displays a high affinity for binding to monoamine transporters (Dewar, et al., 1990; Van Wijngaarden, et al., 1990). Despite its limitations, and because no better selective 5-HT_2 agents are known, [^{3}H]ketanserin is still the most frequently used ligand for studying these receptors (Van Wijngaarden, et al., 1990).

Methysergide The compound methysergide, previously described as an anti-migraine substance, is an antagonist at 5-HT_2 receptors (Peroutka, 1990b). It has been frequently employed by researchers who are further characterizing 5-HT_2 binding sites that have been labeled by [^{3}H]ketanserin (Schoups, et al., 1986, Zifa and Fillion, 1992).

Mesulergine [^{3}H]Mesulergine was found to label 5-HT_2 sites over a decade ago (Zifa and Fillion, 1992). Like methysergide, it is an antagonist at these receptors. It is also used frequently by researchers when investigating 5-HT_2 receptors (Peroutka, et al., 1988; Zifa and Fillion, 1992).

VII. SPECIFIC OBJECTIVES

A. To analyze the 5-HT_{1A} receptor in rabbit cerebral cortex in terms of ligand binding and G protein linkage.

B. To compare values obtained from the characterization of this receptor in rabbit brain with those from rat and human, in order to examine if this receptor exhibits similar characteristics across these three species.

C. To conduct preliminary binding and GTP inhibition studies of solubilized human 5-HT_{1A} receptor, in order to investigate if this receptor maintains the characteristics of membrane bound 5-HT_{1A} receptor.

D. To investigate the interaction of the natural product parthenolide, which has been implicated in the treatment of migraine, with the 5-HT receptors that have gained recognition in treating this ailment.

MATERIALS AND METHODS

I. CHEMICALS

Chemicals, Reagents, and Drugs were obtained from the indicated sources. 8-OH-DPAT, Buspirone, Methiothepin, Propranolol, Spiroxatrine, WB 4101 (Research Biochemicals, Inc.; Natick, MA). [^{3}H]5-HT, [^{3}H]8-OH-DPAT, [^{3}H]Ketanserin, [^{3}H]5-CT (New England Nuclear; Boston, MA). Guanosine 5'-O-(thiotriphosphate) (gamma-S-GTP), [3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate] (CHAPS), n-octyl-b-D-glucopyranoside, Serotonin (5-HT) (Calbiochem; LaJolla, CA). Dopamine, Parthenolide, Sodium cholate (Aldrich Chemical Co.; Milwaukee, WI). Ham's F-12 medium, Fetal Calf Serum (FCS), Earle's Balanced Salt Solution (EBSS), Geneticin (GIBCO/BRL; Gaithersburg, MD). Adenosine 5'-O-(thiotriphosphate) (gamma-S-ATP), Trizma base (Tris), Calcium chloride, Pargyline (Sigma Chemical Co.; St. Louis, MO). Tetrasodium ethylenediamine tetraacetic acid (EDTA), Magnesium sulfate, Ascorbate (Baker Chemical Co.; Phillipsburg, NJ). Chinese Hamster Ovary (CHO) Cells (a gift from Dr. John Raymond, Duke University).

II. MEMBRANE PREPARATION

Brains were removed from New Zealand White rabbits anesthesized with pentobarbital and rapidly dissected at 4° C. After dissection, brain tissue was frozen in liquid

nitrogen and stored at -70° C until the day of use. Cerebral cortex prepared for assay was thawed in 40 volumes of ice-cold buffer (50mM Tris, pH 7.4), minced, and homogenized in a Polytron (setting 5 for 30 seconds). Homogenates were centrifuged at 4° C at 12,000 x g for 45 minutes and pellets were resuspended in an equivalent volume of buffer. This was followed by a second round of homogenization and centrifugation. Rat cerebral cortex was prepared following the same procedure.

III. 5-HT_{1A} SATURATION BINDING ASSAYS

Binding assays were performed using modifications of several techniques that have been previously described (Fargin, et.al.,1988; Nelson, et.al.,1987; Pierce, et.al.,1989; Xiong, et.al., 1989). To assay for binding activity, rabbit or rat membrane pellets were resuspended in 60-80 volumes of Tris buffer containing 10uM pargyline and 4mM calcium chloride (binding buffer). A final assay volume of 1.0 ml contained 0.8 ml of homogenate, 0.1 ml of either binding buffer (representing total binding) or serotonin (10 uM final concentration for non-specific binding), and 0.1 ml of [^{3}H]-8-OH-DPAT (specific activity: 132.8 Ci/mmol) at varying concentrations. Incubations were for 20 minutes at 30° C followed by rapid filtration through glass fiber filters (Whatman GF/B or S & S #32) with three washes of ice-cold Tris buffer (14 mls total). Filters were counted in 5 mls of Ecoscint scintillation fluid in a Beckman Liquid Scintillation System. Tritium was counted at 50%

efficiency. Protein content was analyzed colorimetrically using the Bradford technique (Bradford; 1976).

IV. 5-HT_{1A} DISPLACEMENT EXPERIMENTS

Displacement experiments were identical except that only 0.7 ml of homogenate was used and an additional 0.1 ml of displacing drug (i.e. buspirone, propranolol) at varying concentrations, was added making up the final assay volume of 1.0 ml. The drugs were dissolved at high concentration in distilled water followed by dilution to their final concentrations in binding buffer. As a result of this dilution technique, all drugs were delivered to the assay tubes at virtually identical buffer concentrations as control values. All of the assays were performed in duplicate or triplicate. Each of the displacing drugs was analyzed in at least three separate experiments in rabbit cerebral cortex with the exception of gamma-S-ATP, which was performed twice. The buspirone displacement experiment in rat cerebral cortex was performed only once.

To investigate the possible interactions of the agent used to anesthetize animals (pentobarbital), a single displacement experiment was conducted. The potential displacing agent, pentobarbital, was investigated over a 100 million fold concentration range (10^{-11} M to 10^{-3} M) under identical conditions used in other displacement experiments.

V. HUMAN RECEPTOR

CHO cells that have been transfected with clonal cDNA for the 5-HT_{1A} receptor were grown in culture. Cells were grown in culture at 37° C in a humidified atmosphere of 5% CO_2, 95% air. Cells were cultured in monolayers in 225cm^2 flasks and were nourished with Ham's F-12 medium fortified with 10% FCS and containing 200ug/ml of geneticin. Membrane preparation for binding assays was similar to that for rabbit and rat cerebral cortex. Cells were first washed with 10ml of 0.25% trypsin in physiological saline, and then harvested in an equivalent volume of trypsin. The reaction was stopped by the addition of 10mls of ice-cold F-12 medium containing 10% FCS. Cells were then centrifuged at 4° C at 1,000 x g for 5 min. The resulting pellet was washed with 20mls of Earle's balanced salt solution followed by another round of centrifugation. The pellet was resuspended in 20mls of CHO lysis buffer (20mM Tris, 2mM EDTA, pH 7.4) and homogenized in a Dounce glass homogenizer. The homogenate was centrifuged at 4° C at 12,000 x g for 45 min. The pellet was resuspended in an equivalent amount of lysis buffer and was followed by another round of homogenization and centrifugation. Final membrane pellets were resuspended in 20-25mls of 5-HT_{1A} binding buffer. Incubations involving CHO cells were at 30° C, for 30 min. All dose-response and displacement assays were performed as previously described for rabbit cerebral cortex.

VI. SOLUBILIZED HUMAN RECEPTOR

CHO Cells were harvested in the same manner as previously described for human receptor. Following the final wash, 1ml of solublizing solution containing either CHAPS, Na cholate, or n-octyl-b-D-glucopyranoside (0.6%) was added to the membrane pellet which was allowed to sit on ice for 1 hour. The pellet was then centrifuged at 4° C at 12,000 x g for 15min. The final supernatant was resuspended in 20mls of binding buffer.

VII. PARTHENOLIDE

Because of the non-polar nature of parthenolide, it was necessary to first dissolve the chemical in absolute ethanol before diluting it in binding buffer. Displacement studies at the 5-HT_{1A} receptor were performed in the same manner as previously described. Displacement studies investigating the 5-HT_2 receptor used [^{3}H]ketanserin (specific activity: 60 Ci/mmol) as the radiolabeled ligand which has been shown to bind with high affinity to these receptors (Hoyer et.al.; 1994). Non-specific binding was determined in the presence of 10uM mesulergine in these assays. Displacement assays investigating binding at the 5-HT_{1D} receptor used [^{3}H]5-CT (specific activity: 15 Ci/mmol) as the radiolabeled ligand. Because [^{3}H]5-CT also shows high affinity for 5-HT_{1A} receptors (Hoyer et.al.; 1994), 8-OH-DPAT was used at a concentration of 0.1uM to mask these binding sites. To determine whether parthenolide displaces serotonin from any high affinity sites,

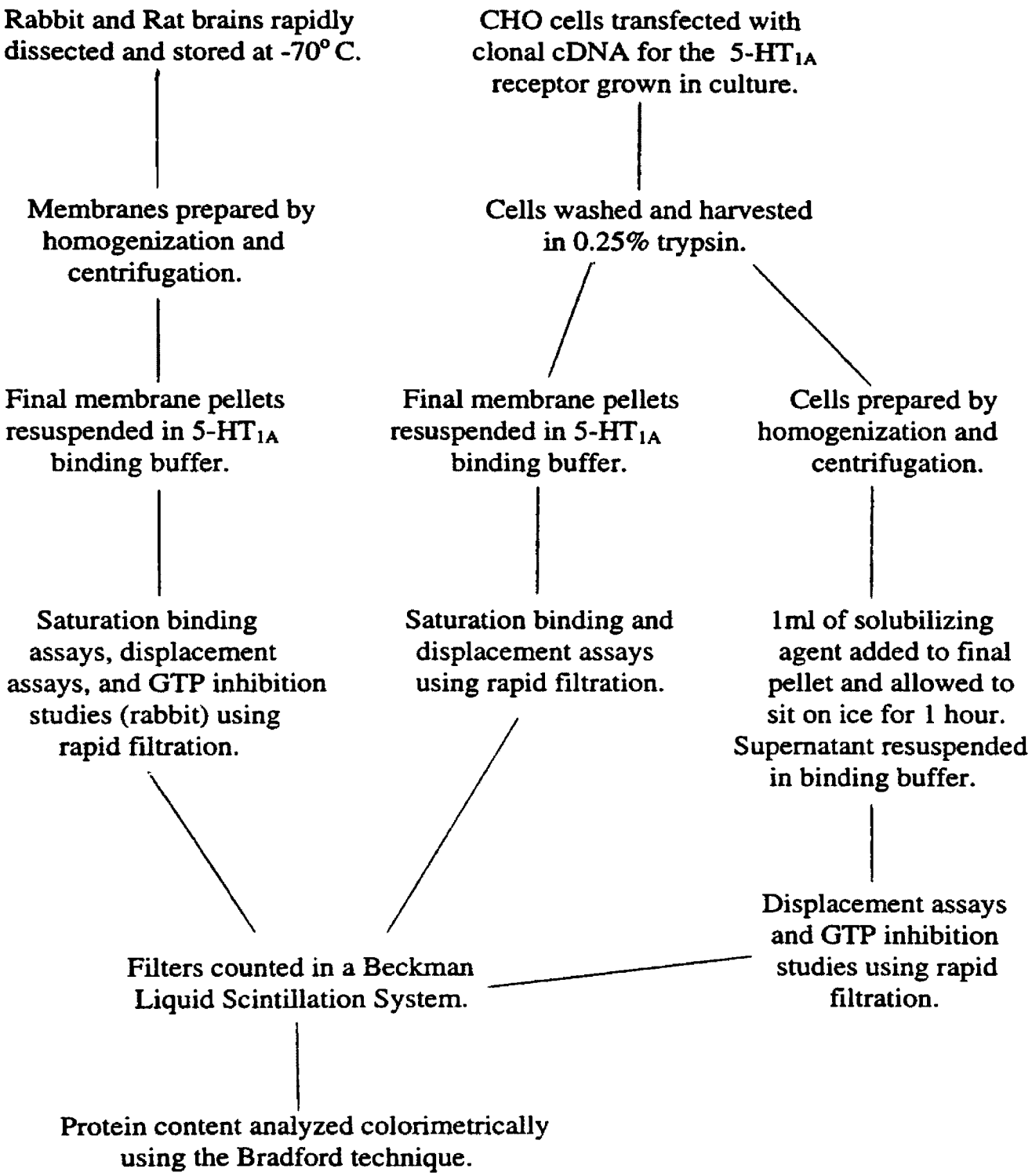

Fig. 5. Flowchart of experimental protocol.

displacement assays utilizing [^{3}H]-5-HT (specific activity: 15 Ci/mmol) were also conducted. Non-specific binding was determined in the presence of 10uM 5-HT when [^{3}H]5-CT and [^{3}H]5-HT were used as the radiolabeled ligands. An experiment was also conducted to investigate the potential interaction of extracts of feverfew with [^{3}H]ketanserin binding. A liquid extract containing 95% ethanol was tested, as well as crude extracts that were reconstituted in pure ethanol.

VIII. STATISTICS

Binding parameters were analyzed by computerized procedures (PC NON-LIN; minitab) as well as fitted by hand. K_i values were determined from IC_{50} values as described by Cheng and Prusoff (1973).

RESULTS

I. 5-HT_{1A} SATURATION BINDING

[^{3}H]8-OH-DPAT, a selective 5-HT_{1A} agonist, showed high affinity binding to rabbit cerebral cortex membranes. Figure 6 shows a representative dose-response experiment in which points were generated in triplicate. When non-specific binding (in the presence of 10uM 5-HT) was subtracted from total binding, a specific, saturable binding process is observed. Using Scatchard analysis of specific binding (Figure 7), an apparent K_d of 1.1 +/- 0.3nM with a B_{max} of 480 +/- 130 fmols/mg protein was obtained (average of 3 experiments; total dose range tested was 0.125nM-5.0nM). Scatchard plots were linear, and Hill plots of the transformed specific binding data (Figure 8) generated a Hill coefficient of 0.96 +/- 0.01, which suggests a single class of non-interacting binding sites. A longer time frame of incubation (30 min) was also investigated, and saturation binding was found to be no better than with a 20 min incubation time. Also, homogenates that were prepared from fresh brain tissue, and then frozen at -80° C, show markedly lower binding activity when they are later tested. Based on these results, and those reported in the literature, the conditions are optimal. [^{3}H]8-OH-DPAT also showed high affinity binding to rat cerebral cortex membranes. Scatchard analysis of specific binding yielded an apparent K_d of 0.8nM in the rat brain (Table 3).

II. 5-HT_{1A} DISPLACEMENT EXPERIMENTS

Figure 9 represents displacement curves in which drugs of known pharmacological specificity competed for binding with [^{3}H]8-OH-DPAT. Drugs with high (spiroxatrine), medium (propranolol) and low (dopamine) affinity for 5-HT_{1A} receptors are shown. K_i's for all displacing drugs that were tested are summarized in Table 2. The buspirone displacement experiment that was conducted in rat cerebral cortex yielded a K_i value of 37 +/- 12 nM (Table 3).

The inhibitory effects of the gamma S analogues of GTP and ATP are summarized in Figure 10. GTP dose-dependently inhibited the binding of specific [^{3}H]8-OH-DPAT to rabbit cerebral cortex membranes. The IC_{50} value for the GTP interaction is 0.3uM. Gamma-S-ATP was essentially without activity.

Maximum displacement achieved with pentobarbital was 70% at a concentration of 1.0 mM. An apparent K_i of 3.0 uM was determined. In a rough calculation using an estimate of the pentobarbital concentration present at anesthesia, the final pentobarbital concentration present in assay tubes following tissue preparation would be less than one-ten thousandth of the pentobarbital K_i. It was estimated that this concentration would affect experimental values for other drugs by less than 5%.

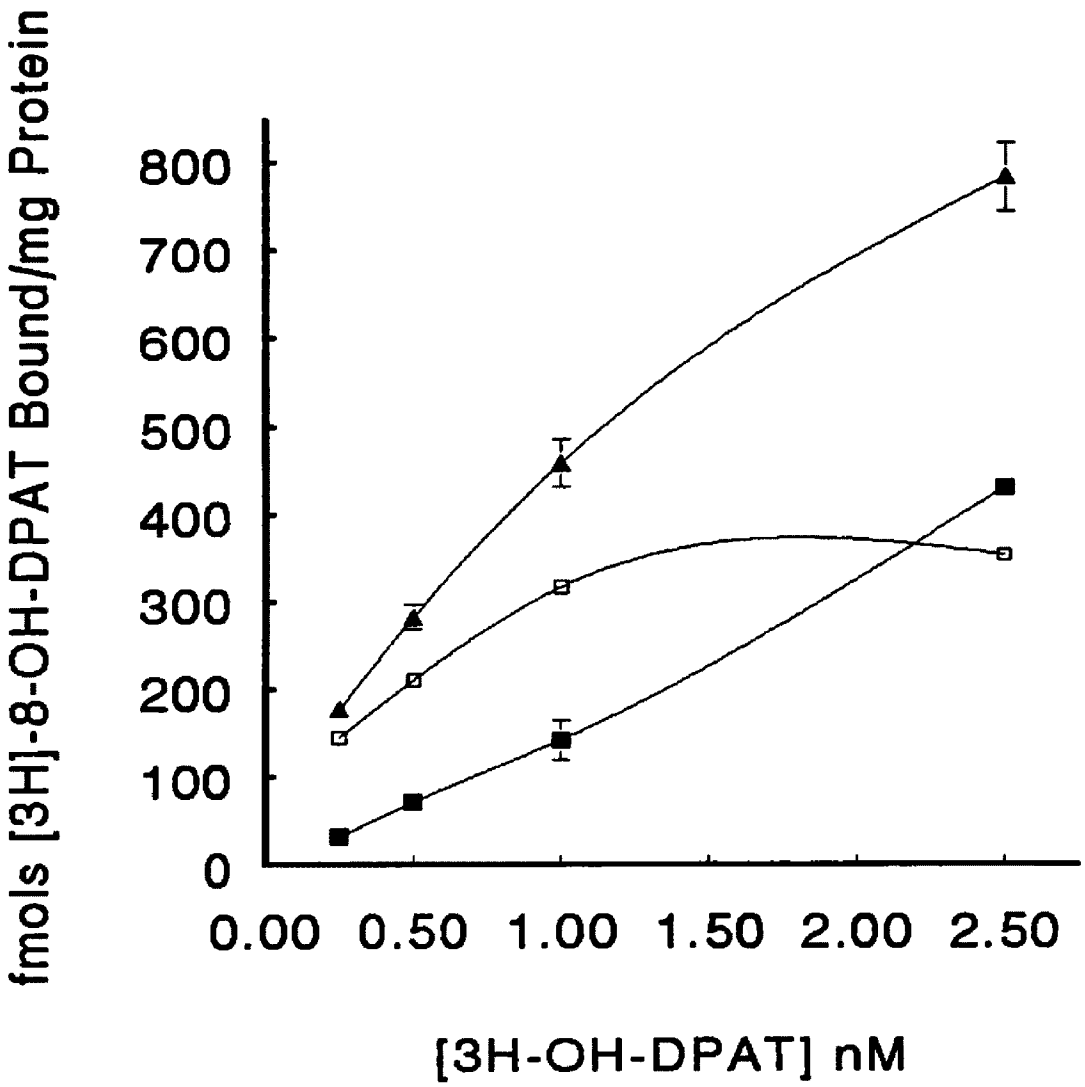

Fig.6. Concentration-dependent binding of [^{3}H] 8-OH-DPAT to membranes from rabbit cerebral cortex. Results are from a representative experiment in which points were generated in triplicate (data shown are mean ± S.E.M.). Specific binding (□) was derived by subtracting non-specific binding (■) from total binding (▲).

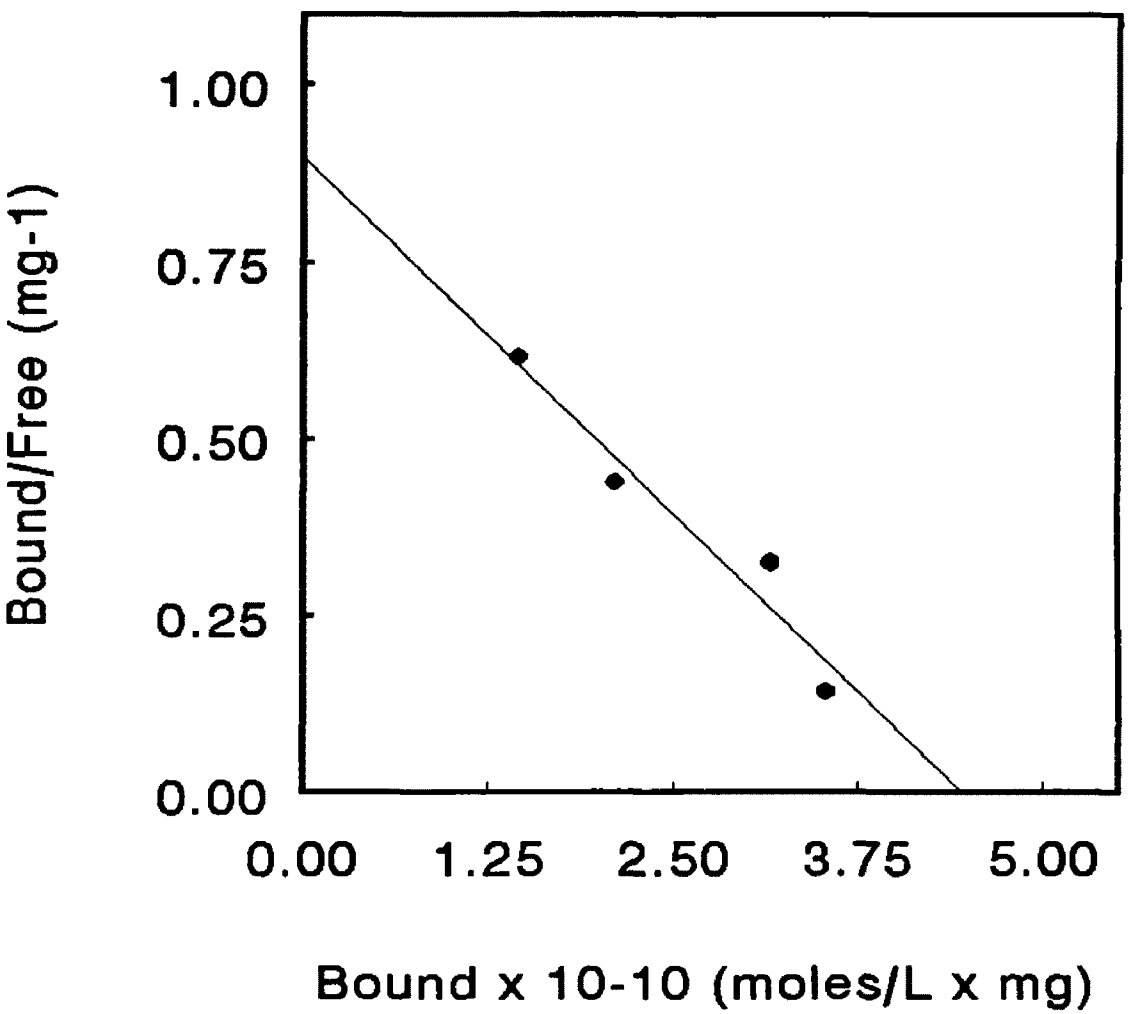

Fig. 7. Scatchard analysis of the binding of [^{3}H] 8-OH-DPAT to 5-HT_{1A} receptors in rabbit cerebral cortex. Results are from a representative experiment in which points were generated in triplicate.

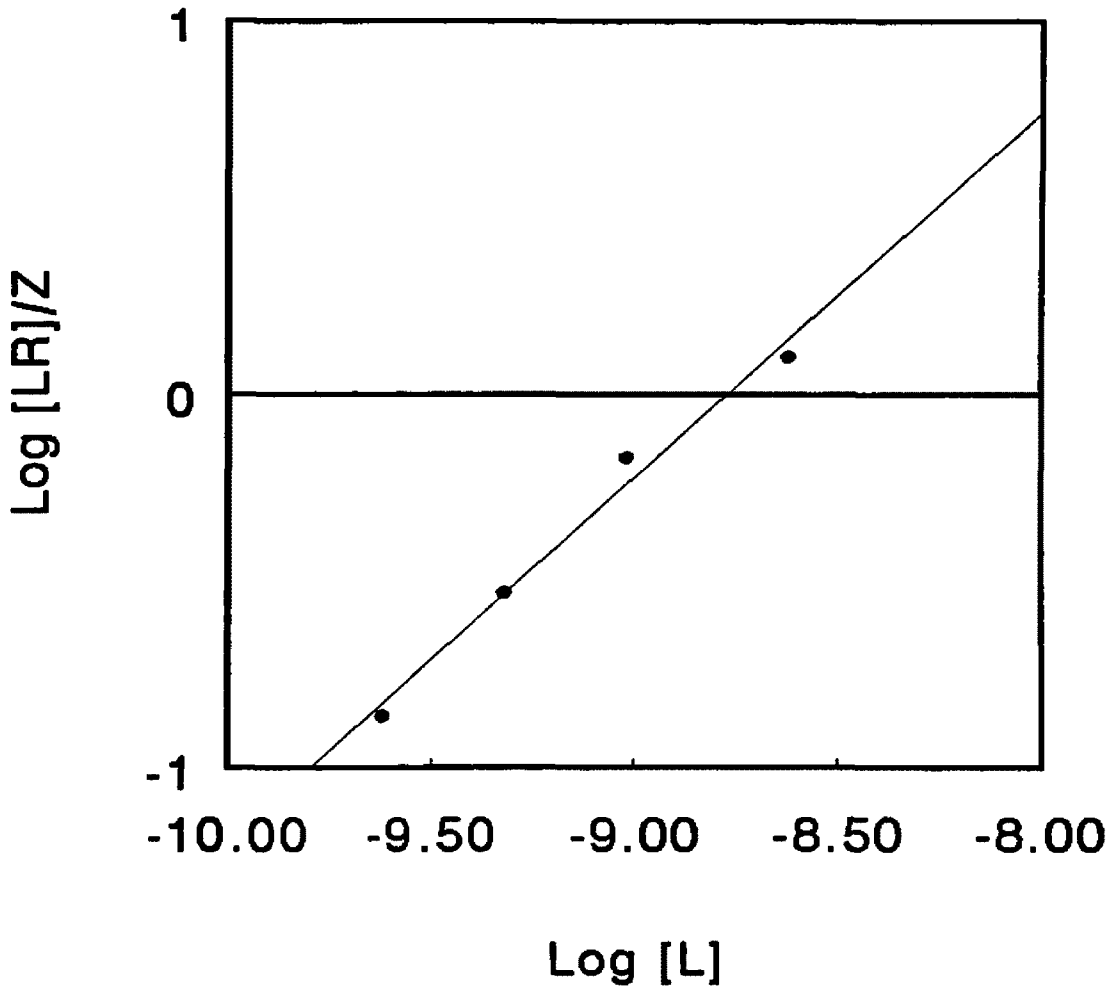

Fig. 8. Hill plot of transformed specific binding data of [^{3}H] 8-OH-DPAT to rabbit cerbral cortex membranes. Results are from a representative experiment in which points were generated in triplicate.[L] represents free ligand. [LR] represents bound ligand. Z= $[LR]_{max}$-[LR].

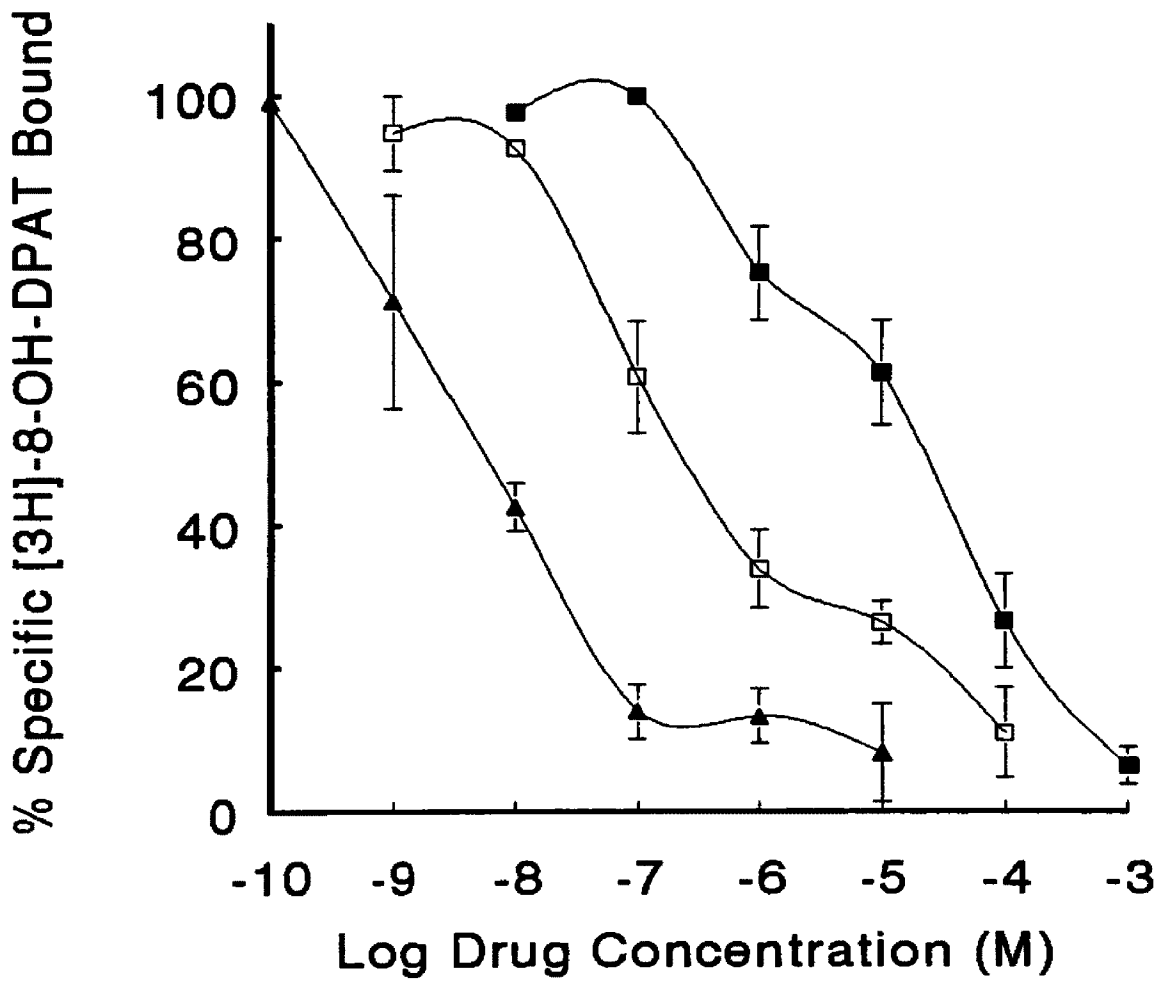

Fig. 9. Displacement of specific [^{3}H] 8-OH-DPAT binding from rabbit cerebral cortex membranes. [^{3}H] 8-OH-DPAT concentration was 0.5nM. Points are mean ± S.E.M. Displacing drugs: spiroxatrine (▲); propranolol (□); dopamine (■).

Table 2 Affinities of ligands for [^{3}H] 8-OH-DPAT binding sites

COMPOUND	K_i (nM)
Spiroxatrine	2.4 ± 0.7
WB 4101	3.0 ± 0.7
Buspirone	26 ± 9
Propranolol	78 ± 2
Methiothepin	807 ± 93
Dopamine	7790 ± 38

Values represent the mean ± S.E.M. for three separate experiments performed in duplicate or triplicate. Experimental conditions and calculations were described in Methods.

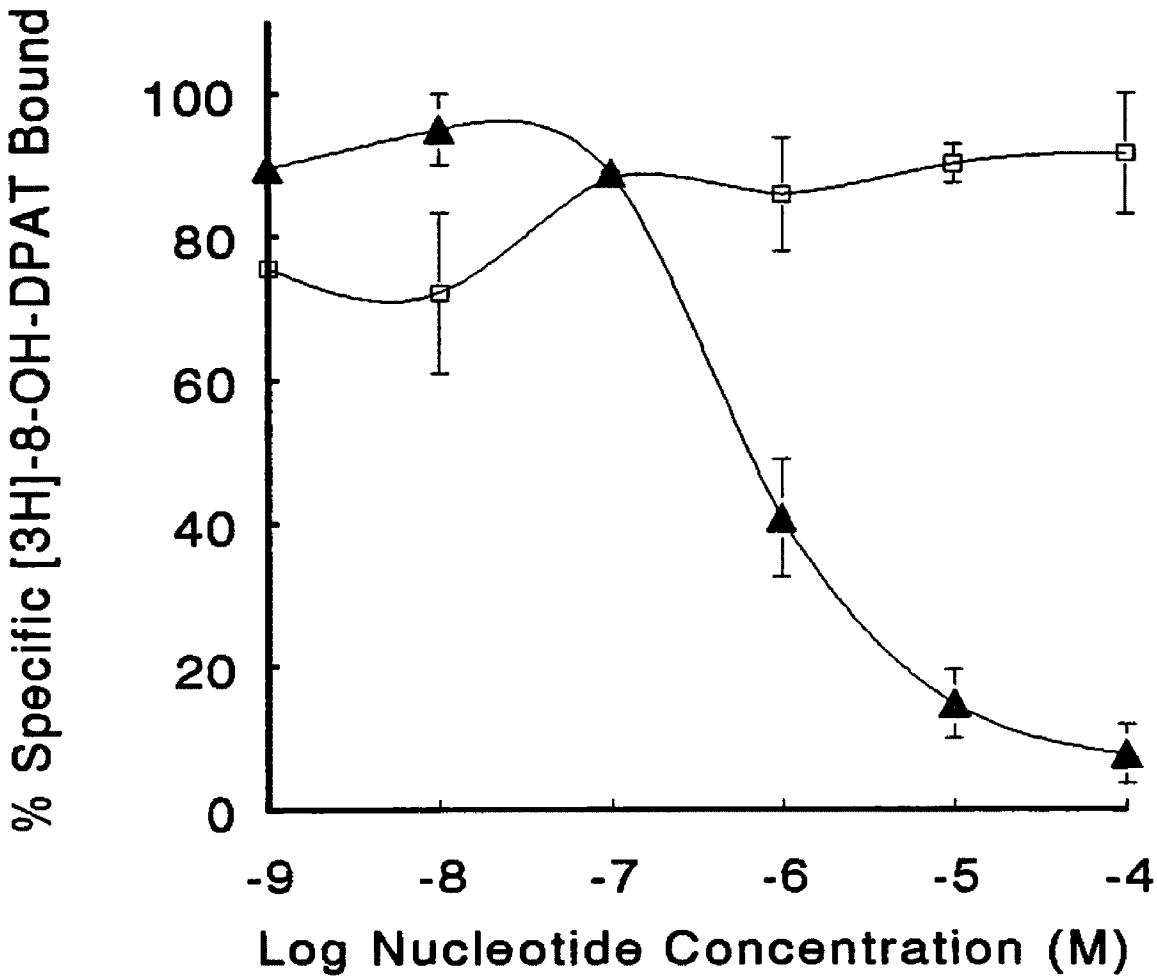

Fig.10. Concentration-dependent inhibitory effect of gamma S GTP and gamma S ATP for [^{3}H] 8-OH-DPAT binding to rabbit cerebral cortex membranes. Gamma S GTP (▲), points are mean ± S.E.M. for three experiments. Gamma S ATP (□), points are mean values from two experiments.

III. HUMAN RECEPTOR

[^{3}H]8-OH-DPAT showed high affinity binding to human 5-HT_{1A} receptor expressed in cultured CHO cells. Figure 11 represents a dose-response experiment in which points were generated in triplicate. Using Scatchard analysis of specific binding, an apparent K_d of 0.6nM with a B_{max} of 770 fmoles/mg protein was obtained (Figure 12). A Hill plot of the transformed specific binding data generated a Hill coefficient of 0.95 (Figure 13), suggesting a non-interacting binding site. Displacement studies of specific [^{3}H]8-OH-DPAT binding with buspirone and propranolol generated K_i values of 5.1 +/- 1.6 nM and 73 +/- 0.2 nM, respectively (Figure 14). Table 3 shows a comparison of saturation binding and displacement data obtained in the rabbit, rat and human.

IV. SOLUBILIZED HUMAN RECEPTOR

Displacement curves generated by competition of buspirone with specific [^{3}H]8-OH-DPAT binding at solubilized human 5-HT_{1A} receptor are shown in Figure 15. Displacement curves at receptors solubilized with both CHAPS and Na cholate are represented. Receptor solubilized with Na Cholate produced a K_i value of 4.3 +/- 0.5 nM for buspirone. Receptor solubilized with CHAPS produced a K_i value of 80 +/- 10 nM. Experiments involving n-octyl-b-D-pyranoside (0.6%) as the solubilizing agent failed to generate a displacement of [^{3}H]8-OH-DPAT by buspirone. Figure 16 shows a

concentration-dependent inhibitory effect of the stable GTP analogue, gamma-S-GTP on specific [^{3}H]8-OH-DPAT binding in human receptor solubilized with CHAPS. The IC_{50} value calculated for the GTP interaction is 0.1uM.

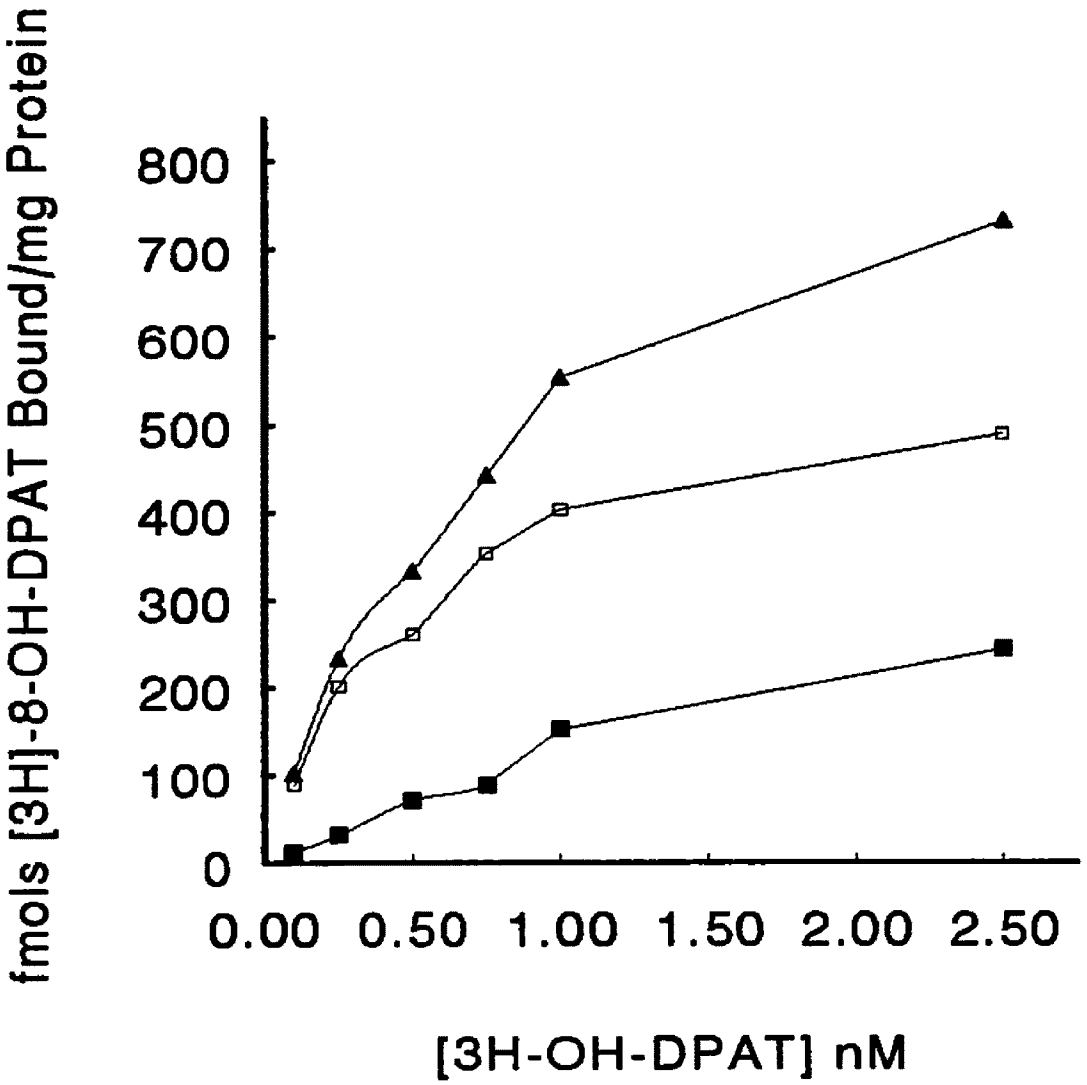

Fig. 11. Concentration-dependent binding of [^{3}H] 8-OH-DPAT to human 5-HT_{1A} receptor expressed in cultured CHO cells. Results are from an experiment in which points were generated in triplicate. Specific binding (□) was derived by subtracting non-specific binding (■) from total binding (▲).

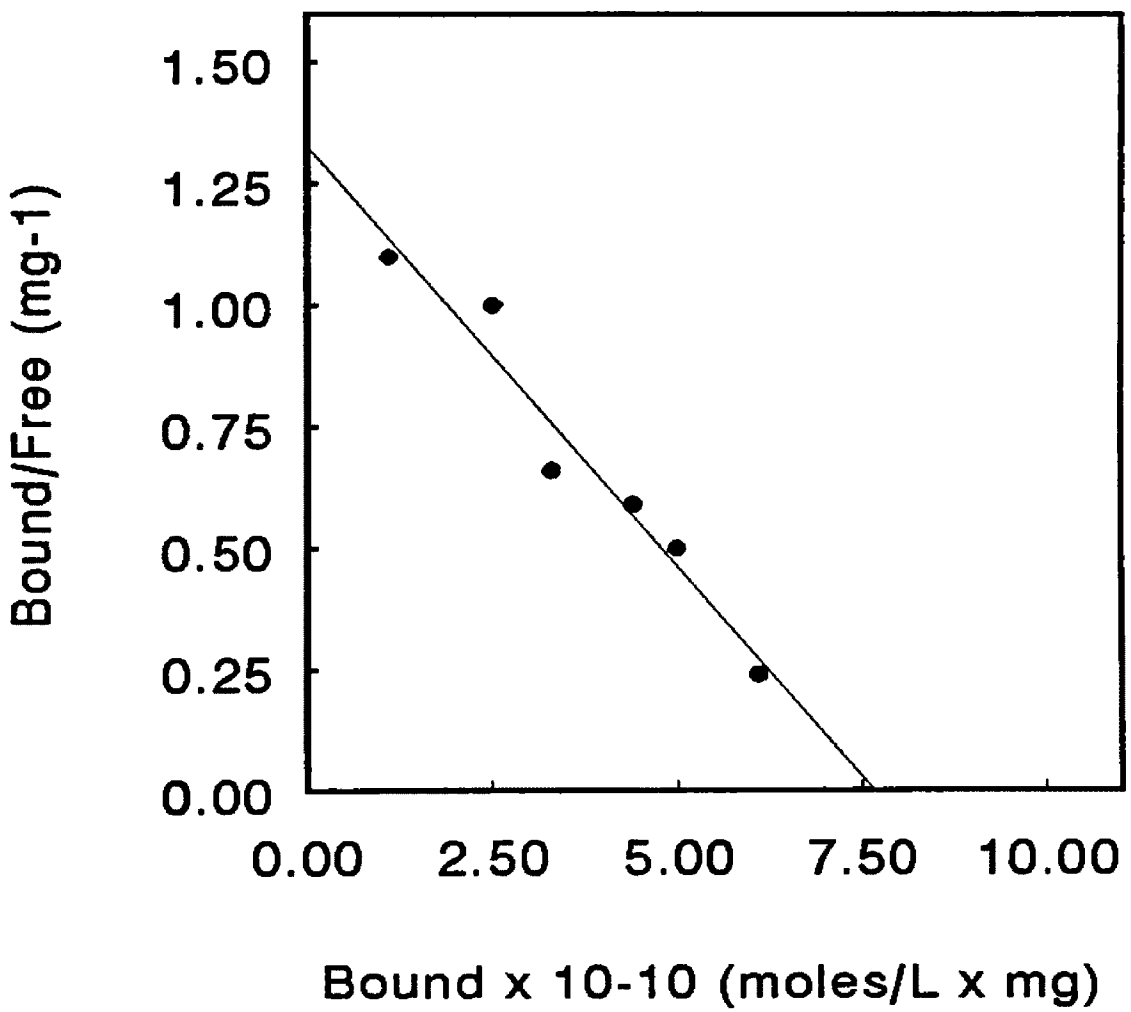

Fig. 12. Scatchard Analysis of the binding of [^{3}H] 8-OH-DPAT to human 5-HT$_{1A}$ receptor expressed in cultured CHO cells. Results are from an experiment in which points were generated in triplicate.

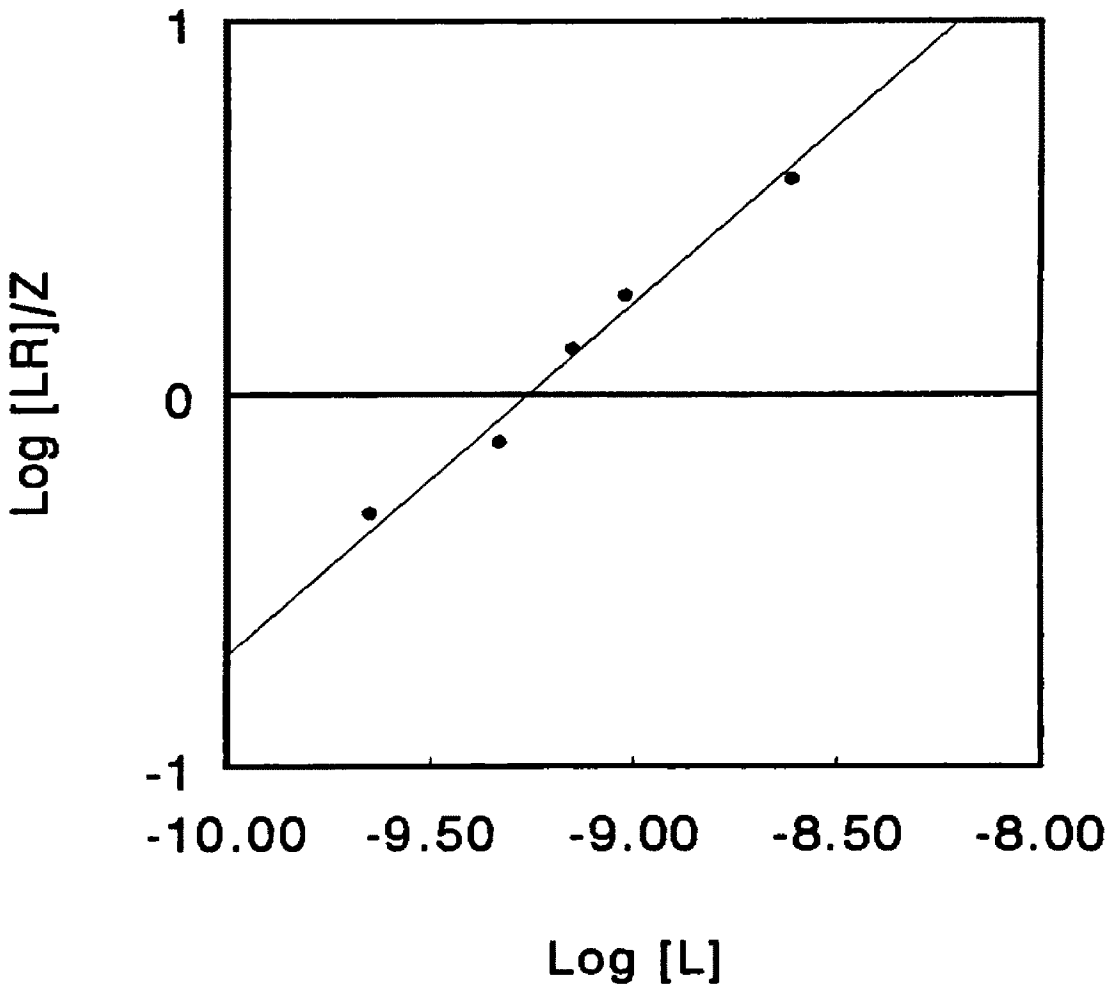

Fig. 13. Hill plot of transformed specific binding data of [^{3}H] 8-OH-DPAT to 5-HT_{1A} receptor expressed in cultured CHO cells. Results are from an experiment in which points were generated in triplicate.[L] represents free ligand. [LR] represents bound ligand. Z= $[LR]_{max}$-[LR].

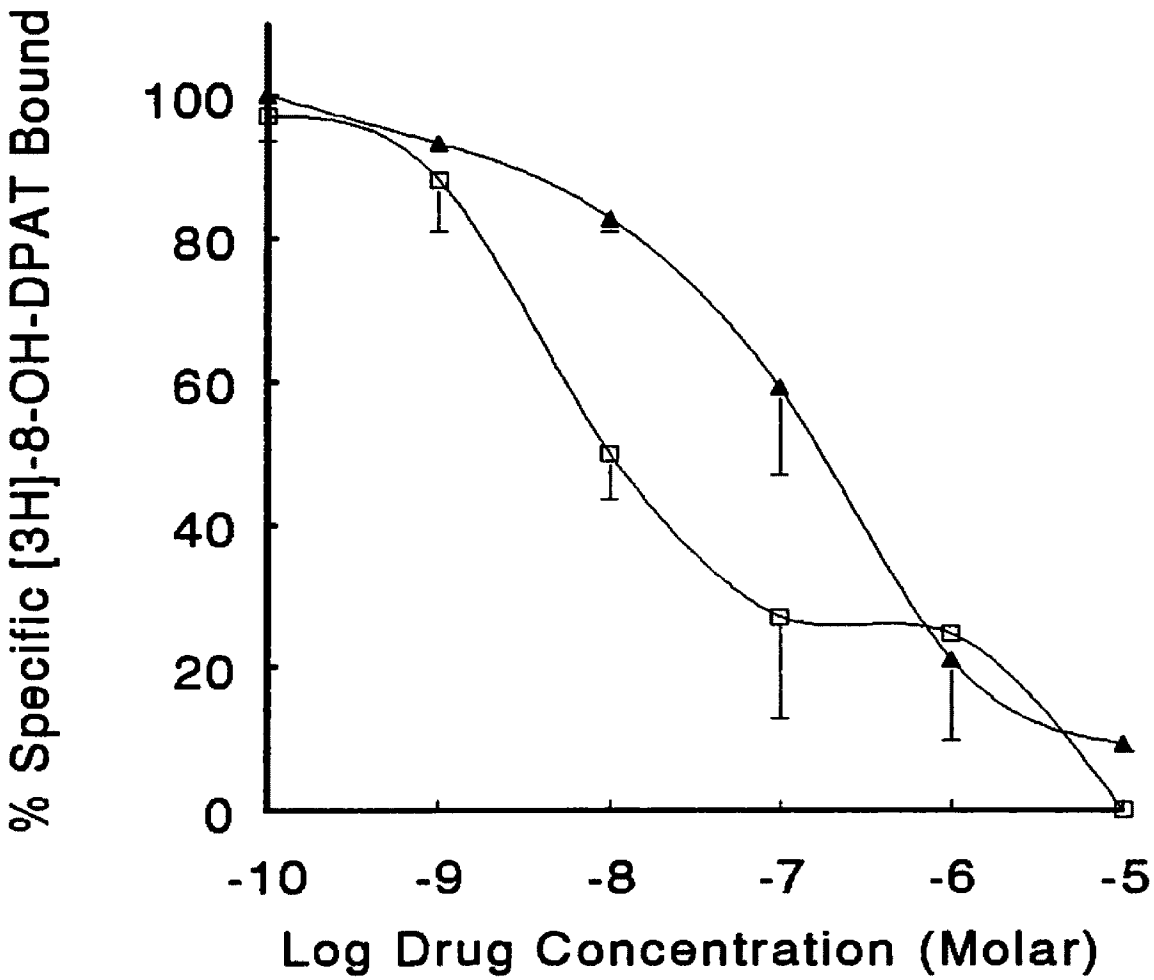

Fig. 14. Displacement of specific [^{3}H] 8-OH-DPAT binding from human 5-HT$_{1A}$ receptor expressed in cultured CHO cells. [^{3}H] 8-OH-DPAT concentration was 0.5nM. Points are mean ± S.E.M. Displacing Drugs: buspirone (□); propranolol (▲).

Table 3 Comparison of Values in Rabbit, Rat and Human

Saturation Binding (Kd)

Rabbit	1.1 ± 0.3 nM
Rat	0.8 nM
Human	0.6 nM

Displacement (K_i)

	Buspirone	Propranolol
Rabbit	26 ± 9 nM	78 ± 2 nM
Human	5.1 ± 1.6 nM	73 ± 0.2 nM
Rat	37 ± 12 nM	

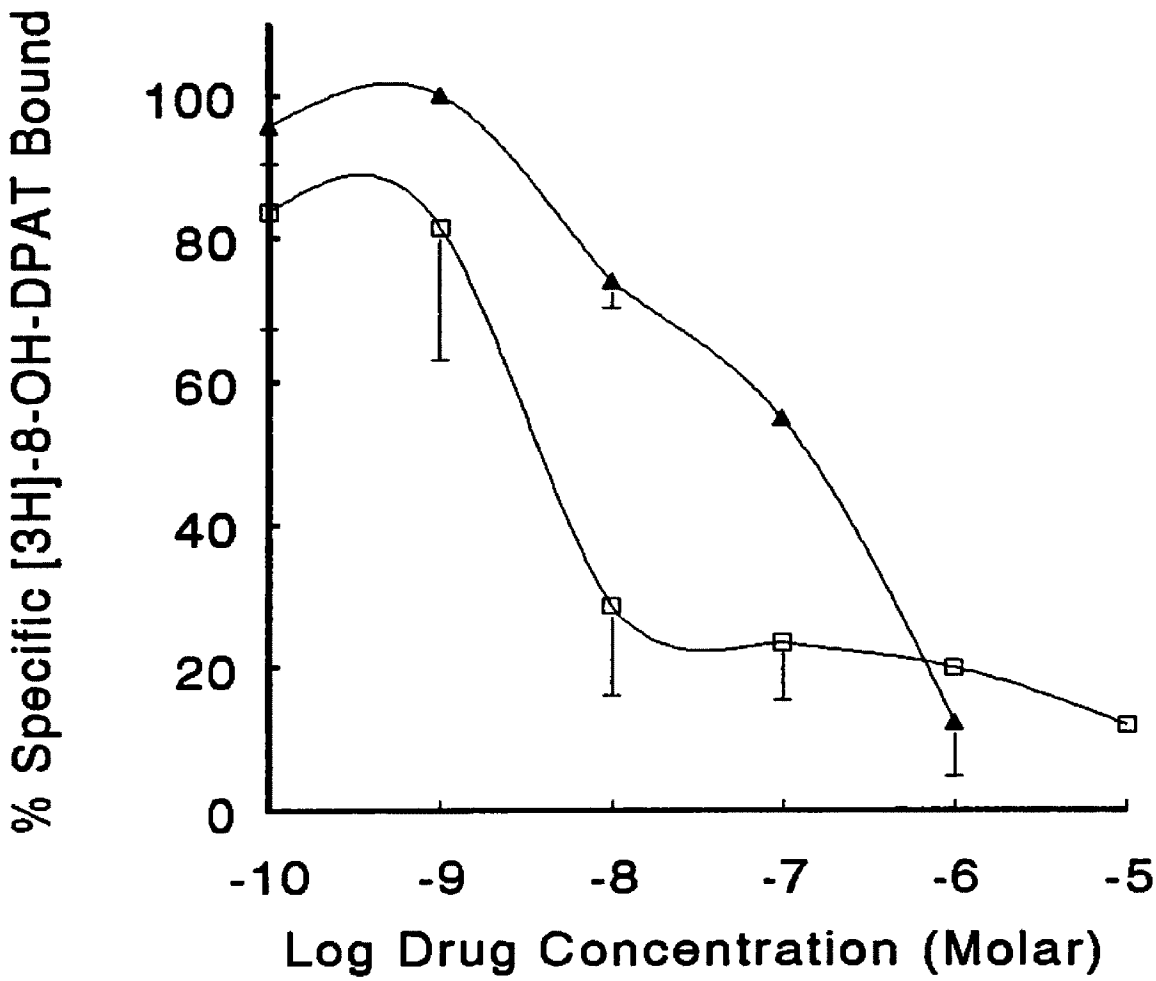

Fig. 15. Displacement of specific [^{3}H] 8-OH-DPAT binding from solubilized human 5-HT_{1A} receptor by buspirone. Points are mean ± S.E.M. Results represent receptor solubilized by CHAPS (▲) and Na cholate (□).

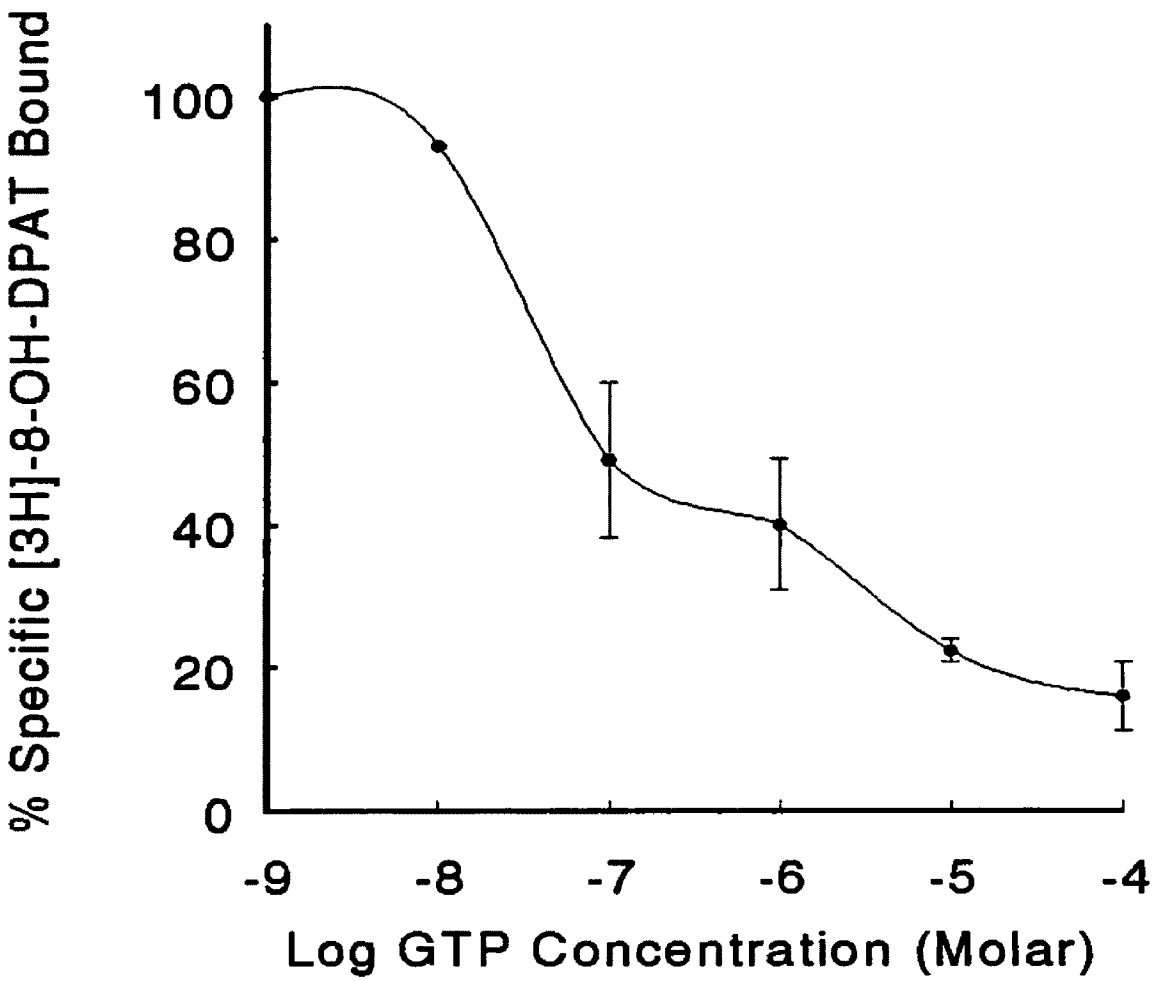

Fig. 16. Concentration-dependent inhibitory effect of gamma S GTP for [^{3}H] 8-OH-DPAT binding to human 5-HT_{1A} receptor solubilized with CHAPS. Points are mean ± S.E.M. for two experiments.

V. PARTHENOLIDE

Comparison of displacement of specific [^{3}H]8-OH-DPAT from rabbit cerebral cortex of parthenolide with a drug of known pharmacological specificity for these receptors, buspirone, is shown in Figure 17. The natural product parthenolide shows very low binding activity at the 5-HT_{1A} receptor, and exhibited a K_i value in excess of 1.0 mM.

Figure 18 represents a comparison of the displacement of [^{3}H]ketanserin by parthenolide and a drug with known specificity at 5-HT_2 receptors, mesulergine. Non-specific binding was determined in the presence of 10uM methysergide in these experiments. Mesulergine produced a K_i value of 8.3 +/- 0.7 nM and parthenolide exhibited an IC_{50} value of exactly 1.0mM. Figure 19 represents a comparison between parthenolide and another drug with known specificity for these receptors, methysergide. Non-specific binding was determined in the presence of 10uM mesulergine in these studies. Methysergide exhibited a K_i value of 5.7 +/- 1.5 nM and parthenolide produced a K_i value of 234 +/- 94 uM. The affinities of these ligands for [^{3}H]ketanserin binding sites are summarized in Table 4.

The experiments involving [^{3}H]5-CT failed to show any displacement of this ligand by parthenolide. Experiments with [^{3}H]5-HT also showed no displacement from its binding sites by parthenolide. Crude extracts of feverfew that were reconstituted in pure ethanol did not displace [^{3}H]ketanserin from its binding sites. However, the liquid extract containing 95% ethanol displaced [^{3}H]ketanserin by 51.3 % in comparison to control values.

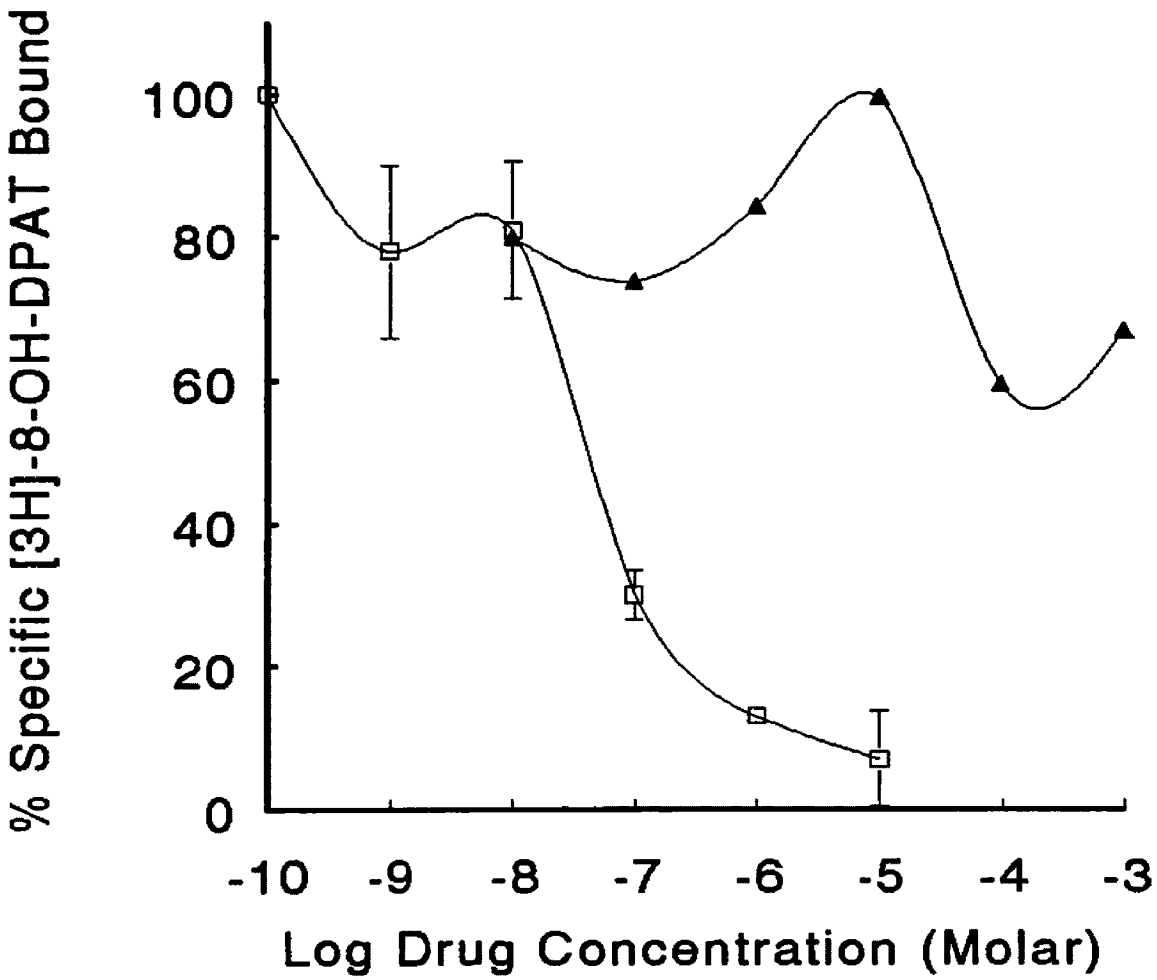

Fig. 17. Displacement of specific [^{3}H] 8-OH-DPAT binding from rabbit cerebral cortex membranes. [^{3}H] 8-OH-DPAT concentration was 0.5nM. Points are mean ± S.E.M. Displacing drugs: buspirone (□); parthenolide (▲).

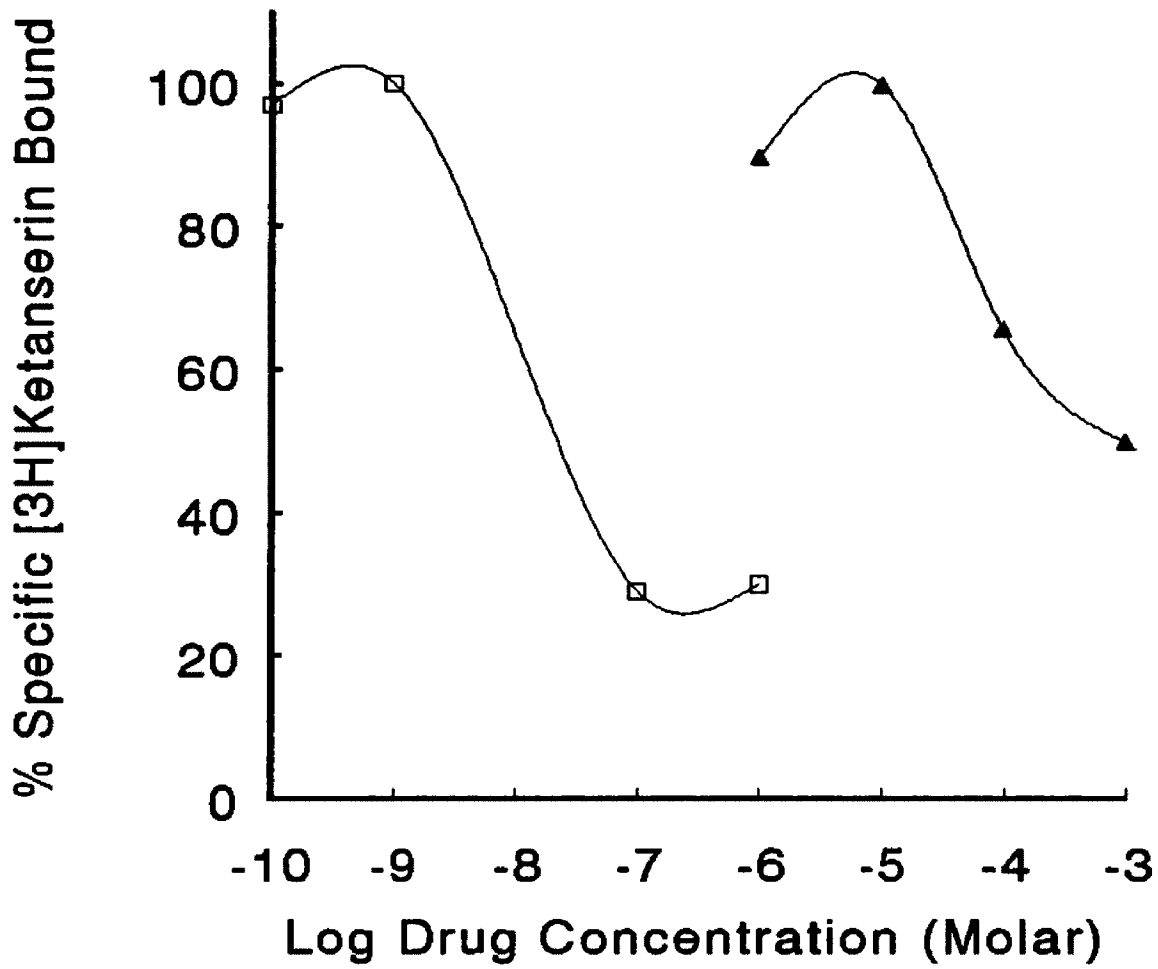

Fig. 18. Displacement of specific [^{3}H] ketanserin binding from rabbit cerebral cortex membranes. [^{3}H]Ketanserin concentration was 0.25nM . Displacing drugs: mesulergine (□); parthenolide (▲).

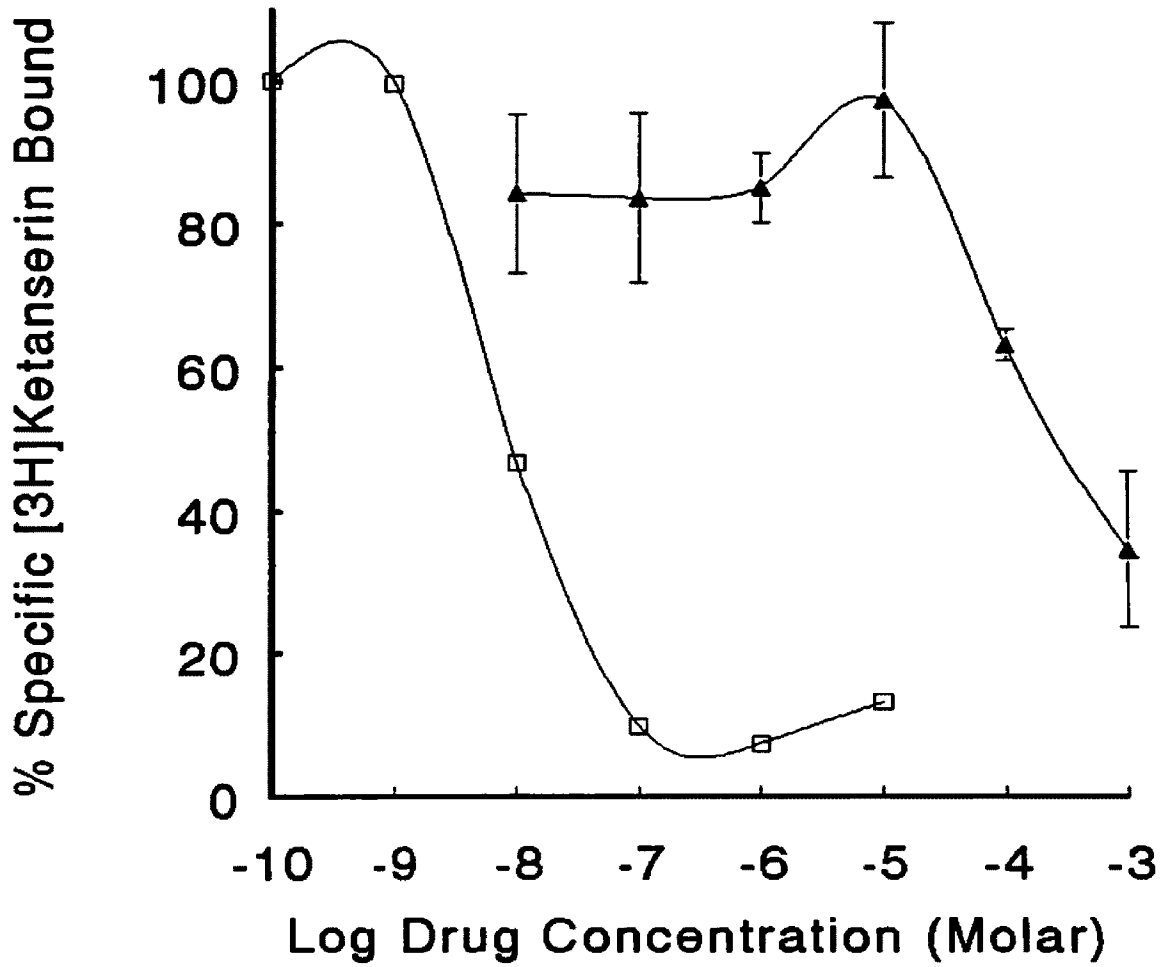

Fig. 19. Displacement of specific [^{3}H] ketanserin binding from rabbit cerebral cortex membranes. [^{3}H] Ketanserin concentration was 0.25nM. Points are mean ± S.E.M. Displacing drugs: methysergide (□); parthenolide (▲).

Table 4 Affininties of ligands for [^{3}H] Ketanserin binding sites

COMPOUND	K_i
Methysergide	5.7 ± 1.5 nM
Mesulergine	8.3 ± 0.7 nM
Parthenolide	234 ± 94 uM

Values represent the mean ± S.E.M. Experimental conditions and calculations were described in Methods.

DISCUSSION

RABBIT BRAIN 5-HT_{1A} RECEPTOR

The 5-HT_{1A} receptor has been identified in several mammalian species (Schnellman, et al., 1984). Although the 5-HT_{1A} receptor has been identified in the rabbit brain (Xiong and Nelson, 1989), it is surprising that the receptor has not been fully characterized in terms of ligand binding and G protein linkage. Based on the results reported here, the 5-HT_{1A} receptor in rabbit brain exhibits similar characteristics in comparison to the 5-HT_{1A} receptor that is found in other mammalian species.

8-OH-DPAT is a highly selective, full agonist at the 5-HT_{1A} receptor. To date, no ligand has been able to surpass the usefulness of [^{3}H]8-OH-DPAT in identifying and characterizing the 5-HT_{1A} receptor (Hoyer, et al., 1994). 8-OH-DPAT showed high affinity binding to membranes from rabbit cerebral cortex. A specific, saturable binding process was observed and produced an apparent K_d value of 1.1nM. This K_d is similar to those reported in other mammalian species with direct binding of 8-OH-DPAT. Reported K_d's range from 0.4 to 3.0 nM, thus suggesting that the rabbit cerbral cortex 5-HT_{1A} binding site is a 5-HT_{1A} receptor similar to 5-HT_{1A} receptors in other species (Beer, et al., 1990; Hoyer, et al., 1994). In addition, Hill plots of the transformed specific binding data produced a Hill coefficient of

0.96 +/- 0.01 , suggesting that this is a single class of non-interacting binding sites. The linear Scatchard plots also support this conclusion that this is a single class of binding sites.

To further characterize this receptor in rabbit cerebral cortex, a series of displacement experiments were conducted in which drugs with known specificity for the 5-HT_{1A} receptor in other species competed for binding with [^{3}H]8-OH-DPAT.

Spiroxatrine exhibited high affinity for the receptor with a K_i value of 2.4 nM. This falls within the range of 0.79 to 7.94 nM that has been reported for other species (Zifa and Fillion, 1992). It is very close to the K_i value of 1.8 nM that was reported by Fargin, et al. (1988) for the clone of the human 5-HT_{1A} receptor. WB 4101 also showed high affinity for the 5-HT_{1A} receptor as has been shown by other researchers (Nelson, et al., 1987; Norman, et al., 1985). The K_i value of 3.0 nM reported here is very close to the value of 5.6 nM that was reported by Harrington, et al. (1991) in rat brain.

K_i values for buspirone at the 5-HT_{1A} receptor have been reported as ranging from 1.2 to 29.5 nM (Zifa and Fillion, 1992). Therefore, the value of 26 nM reported here, is within this range. This value is very close to the 29.5 nM K_i that was reported by Gozlan, et al. (1983) in the rat hippocampus.

The rank order potencies of propranolol (Hoyer, et al., 1994; Sprouse and Aghajanian, 1986) and dopamine (Fargin, et al., 1988; Nelson, et al., 1987) are consistent with 5-HT_{1A} receptors in other mammalian species. The only result that raises questions is that of methiothepin, which produced a K_i of 807nM. The reported range of K_i's for this substance is from 2.2 to 89.1 nM (Zifa and Fillion). This is certainly a large range in

comparison to some of the other ligands used in characterizing this receptor, but the result reported here is almost ten times that of the 89.1 nM K_i reported by Gozlan, et al. (1983). Some of the assay conditions used in previous studies did differ from those that were used in these experiments. For example Norman, et al. (1985), who reported a K_i of 6.7 nM for methiothepin in the rat, used a 40 min incubation time at 37° C. Gozlan, et al. (1983) used an incubation time of only 10 min at 37° C in a 2ml final assay volume. They reported the Ki of 89.1 nM, yet their value for buspirone was very close to the one reported here for the rabbit. Although assay conditions have been shown to differ and can certainly be responsible for some of the differences in reported values, experimental error must be considered for the high K_i value of methiothepin in the rabbit brain. Also, it should be pointed out that methiothepin is not highly specific for this receptor or any other receptor. Therefore, the significance of this reported difference is unclear.

Because 5-HT_{1A} receptors belong to the superfamily of 7 transmembrane G protein coupled receptors, it was necessary to investigate G protein linkage. Guanine nucleotides have been shown to decrease the affinity of agonists for their receptor (Nelson, 1989). Therefore, inhibition experiments utilizing the stable GTP analogue, gamma-S-GTP, were conducted. Gamma-S-GTP dose-dependently disrupted [^{3}H]8-OH-DPAT binding to rabbit cerebral cortex membranes. The IC_{50} value for the reaction was 0.3 uM. As a control, the gamma-S analogue of ATP was also investigated for inhibitory properties. Gamma-S-ATP was essentially without activity. These results are consistent with a receptor which is G protein linked (Fargin, 1988).

To further investigate the characteristics exhibited by the rabbit brain 5-HT_{1A} receptor, studies were conducted to compare binding data obtained in rabbit brain with that obtained from rat brain and cloned human 5-HT_{1A} receptor.

Human 5-HT_{1A} receptor (Fargin, et al., 1988; Kobilka, et al., 1987) was expressed in cultured CHO cells (a gift from Dr. John Raymond of Duke University). When the 5-HT_{1A} receptor is expressed in CHO cultures, it has been found to be negatively coupled to adenylate cyclase (Raymond, et al., 1993). This is the second messenger system that is most often reported in non-transfected systems in other species. Therefore, this receptor appears to exhibit similar characteristics to the 5-HT_{1A} receptors found in other mammalian species, and should serve as a valid model for comparison to the rabbit brain receptor.

[^{3}H]8-OH-DPAT showed high affinity binding to the cloned human 5-HT_{1A} receptor, and upon Scatchard analysis of specific binding, yielded an apparent K_d of 0.6 nM. The Hill coefficient obtained for human receptor was 0.95 suggesting a single site like that in rabbit cerebral cortex. The K_d value of 0.6 nM is close to that obtained in the rabbit, 1.1 nM. The K_d value determined by specific binding of [^{3}H]8-OH-DPAT in the rat brain was 0.8 nM, falling just between the other two values. All three of these values fall within the range of previously reported K_d's (Beer, et al., 1990; Hoyer, et al., 1994), and suggest that a similar binding site is being labeled in the three species.

Displacement values for buspirone and propranolol gave another means of comparing the 5-HT_{1A} receptor across species. The K_i values obtained for propranolol were almost identical in the rabbit brain and for human receptor, 78 and 73 nM, respectively. The

K_i values for buspirone displacement showed more variation. Buspirone showed a higher affinity for the expressed human 5-HT_{1A} receptor with a K_i of 5.1 nM compared to 26 nM for rabbit brain and 37 nM for rat. The Ki value for rat lies outside of the range of previous reported values (Zifa and Fillion, 1992).

The K_i value of 5.1 nM reported here for the buspirone displacement at human receptor is almost identical to the value of 4.0 nM reported by Fargin et al. (1988) for the human clone. Although the transfected human receptor expressed in CHO cells appears to be biochemically similar to the receptor in other species, one cannot forget the fact that the receptor is no longer in its native physiological environment. This may account for the higher affinity that buspirone displayed for this receptor in comparison to rabbit and rat. Certainly, the fact that this receptor is not in its normal environment should always be accounted for.

Another possible explanation for the differing values could be due to the anesthetization of the animals. Although pentobarbital does interact with 5-HT receptors, it is with low affinity, and it is estimated that this effect is minimal. The fact that the K_i values for the propranolol studies in rabbit and the human receptor were almost identical tends to support the idea that pentobarbital is not having a significant affect on the binding data.

The buspirone K_i values for both human 5-HT_{1A} receptor and that in rabbit brain are both within the range of previously reported values (Zifa and Fillion, 1992). The rat K_i value reported here is slightly higher than the previously reported highest value of 29.5 nM in rat hippocampus (Gozlan et al., 1983). Assay conditions used here were different from

those used by Gozlan et al. (1983), and may account for the differences. The high amount of error with the buspirone displacement should also be taken into account, and may be partly responsible for the variations that are seen with the buspirone values.

Viewing previously reported data in the literature and the data presented here, the rabbit brain 5-HT_{1A} receptor seems to exhibit similar characteristics to those reported of the 5-HT_{1A} receptor in other mammalian species. This receptor shows similar characteristics across different mammalian orders, Lagomorpha (rabbit), Rodentia (rat), and Primates (human). The rabbit has often been used as a model when investigating 5-HT receptors in the brain (Dewar, et al., 1990; Feuerstein, et al., 1987; Schoups, et al., 1986; Schoups and DePotter, 1988), yet the 5-HT_{1A} receptor was never fully characterized in this species. Based on these results, the pathology and physiology of this receptor can be confidently studied in the rabbit brain, and data obtained in this species should be applicable to human research.

SOLUBILIZED HUMAN 5-HT_{1A} RECEPTOR

Because certain techniques that are used to investigate receptor-ligand interactions, such as multidimensional NMR, require a large amount of pure receptor, the receptor must be solubilized from its membrane locations. The preliminary experiments reported here were undertaken to investigate if solubilized human 5-HT_{1A} receptor cultured in CHO cells

maintains biochemical and pharmacological binding characteristics comparable to those of membrane anchored receptor.

Displacement studies using buspirone produced a K_i value of 4.3 nM for receptor solubilized with Na cholate, which is very close to the value of 5.1 nM exhibited in nonsolubilized human receptor. Receptor solubilized with CHAPS produced a lower affinity of buspirone for the receptor with a K_i value of 80 nM. Experiments using n-octyl-b-D-pyranoside (0.6%) as the solubilizing failed to show any displacement of [^{3}H]8-OH-DPAT by buspirone. A GTP inhibition experiment was run using CHAPS as the solubilizing agent. GTP dose-dependently inhibited the binding of [^{3}H]8-OH-DPAT to solubilized human 5-HT_{1A} receptor. An IC_{50} value of 0.1uM was determined for the interaction.

Based on this preliminary data, the solubilized receptor seems to maintain its biochemical and binding characteristics after its removal from the membrane. Buspirone certainly had a higher affinity for receptor solubilized by Na cholate, but because of its compatibility with techniques like NMR , CHAPS was utilized in the GTP experiment. The result produced by this experiment suggests that the receptor is remaining G protein linked after solubilization. Certainly, many more experiments are needed, but these preliminary results suggest that the qualities of the 5-HT_{1A} receptor expressed in a tissue culture system can be maintained after solubilization with detergents.

PARTHENOLIDE

Parthenolide showed very low activity at the 5-HT_{1A} receptor. A K_i value in excess of 1.0 mM was obtained, in comparison to a drug with known high affinity for this receptor, buspirone, which exhibited a K_i value of 26 nM. Parthenolide did not exhibit any affinity at the 5-HT_{1D} receptor, as it failed to displace [^{3}H]5-CT from these binding sites. These results are not entirely suprising, as feverfew has generally been used in the prevention of migraine attacks (Awang, et al., 1991). Based on the hypothetical involvement of 5-HT receptors in migraine pharmacotherapy, an interaction at 5-HT_2 receptors would seem to be more expected for a compound working as a prophylactic medicine for migraine.

The interaction of parthenolide at 5-HT_2 receptors was investigated by comparing the displacement of [^{3}H]ketanserin with compounds of known affinity for these binding sites, methysergide and mesulergine. Methysergide, a 5-HT_2 antagonist that has been employed as an anti-migraine drug, exhibited a K_i of of 5.7 nM, and thus high affinity for the 5-HT_2 receptor in rabbit cerebral cortex. This value falls well within the range reported by other researchers for mammalian species, from 0.94 to 12 nM (Leysen, et al., 1982; Zifa and Fillion, 1992). It is exactly the 5.7 nM value that was reported by Peroutka (1990). High affinity binding to 5-HT_2 sites was also shown by mesulergine, which exhibited a K_i of 8.3 nM. This value also correlates well with those reported of other researchers (Zifa and Fillion, 1992). Because of the non-polar properties of parthenolide, it was necessary to first dissolve the chemical in absolute ethanol before further diluting in the assay buffer. A control experiment was run to investigate the potential interaction of ethanol with 5-HT_2

receptors. Ethanol was tested at the highest concentration in which appeared in the final assay volume (2.0%), and it alone failed to disrupt any binding of [^{3}H]ketanserin. Therefore, assay conditions appear to be appropriate for the analysis of the interactions of parthenolide with these receptors. When mesulergine was used for non-specific binding, parthenolide produced an IC_{50} value of exactly 1.0mM. When methysergide represented non-specific binding, a K_i of 234 nM was obtained. Therefore, parthenolide is displacing [^{3}H]ketanserin from its binding sites, but with very low affinity, and certainly is not compatible with the high affinities that are displayed by the 5-HT_2 antagonists, methysergide and mesulergine.

Experiments were also conducted using [^{3}H]5-HT to examine if parthenolide was displacing serotonin from any high affinity sites and to determine if any other 5-HT receptors may possibly be involved in the action of this substance. No displacement of [^{3}H]5-HT was exhibited. Based on these results, parthenolide does not appear to exhibit its reputed effects by acting directly at 5-HT receptors. A few conclusions can be made.

The proposed hypothesis to explain the efficacy of 5-HT_2 receptor antagonists in treating migraine is as follows. These receptors have been found to mediate contraction of smooth muscles in the vasculature (Peroutka, 1984), and it has also been shown that 5-HT can stimulate the production of prostacyclin and other arachidonic acid metabolites in smooth muscle (Coughlin, et al., 1981; Coughlin, et al, 1984). 5-HT_2 antagonists, like methysergide, have been demonstrated to block these effects. Therefore, they appear to be mediated through a 5-HT_2 mechanism. Because 5-HT_2 receptors are positively linked to phospholipase C, and phospholipase C can stimulate arachidonic acid metabolism, this adds

support to the hypothesis. The importance of these findings are that modulation of arachidonic acid metabolism may have important effects on vascular tone, and 5-HT could stimulate arachidonic acid metabolism at the beginning of a migraine attack, via 5-HT_2 receptors. This could lead to an inflammatory reaction in the brain vasculature, and theoretically, 5-HT_2 antagonists are able to inhibit 5-HT from inducing this inflammatory state. Once this inflammatory reaction has begun however, 5-HT_2 antagonists would be of little benefit (Peroutka, 1990a).

Parthenolide may work by a mechanism that is associated with this hypothesis. This compound has been shown to inhibit the release of 5-HT from blood platelets (Groenewegen and Hepinstall, 1990; Marles, et al., 1992). Perhaps, through this mechanism alone, it is inhibiting the induction of this inflammatory state by blocking 5-HT release, which would otherwise stimulate arachadonic acid metabolism. Importantly, extracts of feverfew have been shown to inhibit both 5-lipoxygenase and cyclo-oxygenase pathways of endogenous arachidonic acid metabolism (Sumner, et al., 1992). It may block an inflammatory reaction leading to a migraine attack by inhibiting these enzymes. Still, it may be a combination of this latter mechanism with the inhibition of 5-HT from platelets that produces the anti-migraine effects.

Another possibilty is that, due to the difficulties of getting parthenolide into solution, there may not be enough of the substance in the assay volume to show a significant amount of binding to receptors. Every effort was made to insure that parthenolide was indeed in solution. An experiment was also run in the presence of a solubilizing agent, but it

failed to show any improvement of the displacing of [^{3}H]ketanserin by parthenolide. The possibilty that parthenolide was not fully in solution should not be completely ruled out.

Still, another possibility is that some other chemical in the plant or a combination of parthenolide and other chemicals, lead to the efficacy of feverfew. Sumner, et al. reported (1992) that parthenolide was a potent inhibitor of arachadonic acid metabolism, but they also found that extracts which did not contain parthenolide inhibited this metabolism as well. Therefore, the presence of parthenolide is not necessary for this effect. Other investigators have shown that dried and fresh leaves of feverfew have different pharmacological profiles on vascular smooth muscle (Barsby, et al., 1992). Dried leaves lack some of the effects that are seen in fresh leaves and therefore, it may be advantageous to ingest fresh feverfew leaves for maximum efficacy. These effects were attributed to sequiterpene lactone content, which includes parthenolide.

Therefore, questions arise as to whether or not parthenolide has potential alone as an anti-migraine agent, or if it is necessary to ingest the leaves of feverfew to produce desired effects, which have been verified in clinical trials (Johnson, et al., 1985; Murphy et al., 1988). In light of these findings, an experiment was conducted in which crude extracts of parthenolide were investigated for their potential of displacing [^{3}H]ketanserin binding. Crude extracts that were reconstituted in pure ethanol did not displace [^{3}H]ketanserin binding. However, a liquid extract of 95% ethanol displaced [^{3}H]ketanserin binding by approximately 50%. This result suggests that some polar component of the plant may be binding to 5-HT_2 receptors, or that a combination of chemicals is producing a displacement

of [^{3}H]ketanserin binding. This finding is very preliminary and crude at best, but it does suggest that further experiments investigating the potential interaction of the products of feverfew with 5-HT receptors, are warranted.

CONCLUSIONS:

A. The 5-HT_{1A} binding site in the rabbit brain exhibits similar characteristics to those found of 5-HT_{1A} receptors in other mammalian species. The 5-HT_{1A} site in rabbit brain appears to be G protein-linked, a characteristic found of the 5-HT_{1A} receptor in other species. The rabbit 5-HT_{1A} site also exhibits similar binding characteristics in comparison to rat and human 5-HT_{1A} receptor. Therefore, the physiology and pathology that is associated with the 5-HT_{1A} receptor can be studied confidently in the rabbit brain.

B. In preliminary studies, human 5-HT_{1A} receptor that is expressed in CHO cells appears to maintain the qualities of membrane-bound receptor after solubilization with detergents.

C. The potential anti-migraine substance, parthenolide, does not appear to interact with 5-HT_{1A} or 5-HT_{1D} receptors. Parthenolide does bind to 5-HT_2 receptors, but with low affinity. Therefore, parthenolide does not appear to produce its hypothesized anti-migraine effects by binding directly to 5-HT receptors in the brain. Parthenolide may produce anti-

migraine effects through another mechanism. Another possibility is that some other chemical in the feverfew plant is responsible for these effects, or that parthenolide in combination with other constituents in the plant are responsible for its effects.

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