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CHARACTERIZATION OF TWO 5-HYDROXYTRYPTAMINE RECEPTORS COUPLED TO ADENYLATE CYCLASE IN GUINEA PIG HIPPOCAMPUS

by

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ABSTRACT

CHARACTERIZATION OF TWO 5-HYDROXYTRYPTAMINE RECEPTORS COUPLED TO ADENYLATE CYCLASE IN GUINEA PIG HIPPOCAMPUS

by

ANDREW SHENKER

Advisor: Jack Peter Green, M.D., Ph.D.

The development of appropriate assay conditions has allowed quantitiative pharmacological characterization of 5-HT-stimulated adenylate cyclase activity in membranes of adult guinea pig hippocampus. Stimulation of adenylate cyclase activity by 5-HT is concentration-dependent, GTP-dependent, and additive to responses elicited by dopamine, (-)isoproterenol and histamine, indicating that distinct 5-HT receptors mediate the effect.

Treatment of guinea pigs with reserpine, which depletes brain 5-HT, selectively increases the responsiveness of hippocampal adenylate cyclase to 5-HT. Exposure of hippocampal membranes to 5-HT in vitro causes decreased responsiveness to 5-HT. Maximal stimulation by 5-HT may be related to the proportions of coupled and desensitized 5-HT receptors in the preparation.

Characterization of 5-HT-stimulated adenylate cyclase activity reveals that two, pharmacologically distinct 5-HT

receptors mediate the response. The two receptors were characterized with selected agonists and antagonists and with the aid of computerized curve-fitting procedures. Neither receptor may be classified as the 5-HT_2 or as the "peripheral neuronal" (5-HT_3) type. 5-HT is only about 10-fold more potent in eliciting response through one cyclase-linked receptor (R_H) than the other (R_L). The two receptors are best discriminated by the agonists 5-carboxamidotryptamine and 8-hydroxy-2(di-n-propylamino)-tetralin and by the antagonist spiperone, all of which are selective for R_H. Spiperone acts as a simple competitive antagonist at R_H, with a dissociation constant of 20 nM. The characteristics of R_H suggest that it is the functional correlate of the 5-HT_{1A} binding site in brain; this receptor may mediate some of the behavioral and electrophysiological effects of 5-HT in mammals. The atypical anxiolytic buspirone is a potent partial agonist at R_H, a finding that may be useful in understanding the pharmacological and therapeutic effects of this drug.

R_L probably does not correspond to a known 5-HT binding site, but it may be homologous to receptors that mediate 5-HT-stimulated adenylate cyclase activity in other systems, especially in infant rat colliculi.

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INTRODUCTION

5-Hydroxytryptamine (5-HT, serotonin) is believed to play a role in physiological and pathological processes in the cardiovascular, gastrointestinal, and nervous systems of mammals (Douglas, 1980; Vanhoutte et al., 1984; Fuller, 1984). Various drugs, including antidepressants (Peroutka and Snyder, 1980), hallucinogens (Aghajanian, 1981; Jacobs, 1983) and antimigraine agents (Fozard, 1982), are known to act on serotonergic systems. Although the existence of multiple types of receptors mediating responses to 5-HT was first suspected thirty years ago (Gaddum and Hameed, 1954; Gaddum and Picarelli, 1957), many 5-HT receptors, including those linked to adenylate cyclase, remain unclassified (Wallis, 1981; Humphrey, 1983). Categorization of mammalian 5-HT receptors lags behind that of adrenergic, cholinergic, and histaminergic receptors. Classification of the receptors for 5-HT could increase understanding of the physiological functions of 5-HT and facilitate the development of new, selective therapeutic agents.

Several factors may account for the delay in establishing a definitive taxonomy of 5-HT receptors: (1) Many of the effects of 5-HT that have been described *in vivo* or *in situ* (e.g., changes in blood pressure, electrophysiological, behavioral and endocrinological effects) are not ideal for quantitative receptor characterization. Drug distribution and metabolism, reflex

mechanisms, and physiological antagonism in intact preparations may obscure information about drug-receptor interactions.

(2) In some cases, experiments may not have been designed and performed in a way to yield data suitable for 5-HT receptor characterization. Multiple receptors for other biogenic amines have been unambiguously defined by determining the relative potencies of agonists and the affinities of competitive antagonists in isolated organ preparations and broken-cell adenylate cyclase assays. The care that must be taken to guarantee satisfactory assay conditions has been discussed (Furchgott, 1972; Kenakin, 1982). Factors that can produce misleading data, even in "simple" serotonergic systems, include agonist uptake and metabolism (e.g., Vane, 1959; Ireland et al., 1983; Bradley et al., 1984) and interaction of agonists with non-5-HT receptors, including beta-adrenergic receptors (Edvinsson et al., 1978), alpha-adrenergic receptors (Apperley et al., 1976; Stollak and Furchgott, 1983), and nicotinic receptors (Wallis, 1981). Experiments should be designed so that "the full range of tests for competitive antagonism" (Black et al., 1982) can be conducted. Classifying receptors on the basis of IC_{50} values, or on the basis of inhibition produced by only one concentration of antagonist may lead to confusion, especially if the antagonist acts noncompetitively.

(3) In fact, so-called "classical" 5-HT antagonists often do not behave as simple competitive antagonists; this precludes estimation of their receptor affinity. The antagonism of 5-HT response by drugs such as LSD, methysergide, and cyproheptadine is non-surmountable in a variety of preparations, including cranial blood vessels (Muller-Schweinitzer, 1976; Edvinsson et al., 1978; Peroutka et al., 1983), coronary artery (Brazenor and Angus, 1981; Frenken and Kaumann, 1984), dog saphenous vein (Apperley et al., 1980), rat stomach fundus (Offermeier and Ariens, 1966), rabbit cornea (Neufeld et al., 1982), rabbit aorta (Black et al., 1984), and homogenates of infant rat colliculi (Enjalbert et al., 1978b). The noncompetitive behavior of cyproheptadine in some preparations may be explained by its blockade of calcium channels (Peroutka and Allen, 1984), while the non-surmountable antagonism produced by LSD may be due to its complex interaction with the 5-HT receptor (Black et al., 1984).

(4) Many responses to 5-HT undergo rapid tachyphylaxis. Tachyphylaxis, or desensitization, is a phenomenon in which the response of a target tissue is attenuated after exposure to an agonist; in many cases, this process apparently involves selective inactivation of receptors (e.g. Lefkowitz et al., 1980). One of the first reports of response to 5-HT in guinea pig ileum (Gaddum, 1953) also noted the rapid and specific tachyphylaxis of that response. Subsequently, desensitization of response to 5-HT was described in a

variety of systems (Vane, 1957; Fozard and Mobarok Ali, 1978a; Huidoboro-Toro and Foree, 1980; Fillion et al., 1980b; Wallis, 1981; Lemberger et al., 1984). In fact, for many years, 5-HT itself was considered the most useful "antagonist" available for neuronal 5-HT receptors. Desensitization of a response to 5-HT may hinder the pharmacological characterization of the receptors mediating that response.

(5) The complex effects of 5-HT in certain preparations may be due to the presence of multiple types of 5-HT receptors, mediating similar or opposite effects (e.g., vasoconstriction plus vasodilatation) (Vargaftig and Lefort, 1974; Eyre, 1975; Vanhoutte et al., 1984; Verdouw et al., 1984). In pig coronary artery, for example, an endothelium-dependent relaxation response to 5-HT is "unmasked" by treating the tissue with an antagonist of the 5-HT receptors that mediate contraction (Cocks and Angus, 1983).

In spite of these problems, the availabilty of potent and selective antagonists has recently permitted the definition of two classes of functional 5-HT receptors in mammals.

I. Classification of Mammalian 5-HT Receptors

Gaddum and co-workers (Gaddum and Hameed, 1954; Gaddum and Picarelli, 1957) presented the first classification scheme for multiple 5-HT receptors, which were termed M and

D, based on their respective sensitivity to morphine and dibenzyline in guinea pig ileum. Unfortunately, neither morphine nor dibenzyline are specific antagonists of 5-HT receptors; other studies (Barlow and Khan, 1959; Day and Vane, 1963; Costa and Furness, 1979; Chahl, 1983) have emphasized that the action of 5-HT in the guinea pig ileum is complex. The prototypic M and D receptors in the ileum remain incompletely characterized (Humphrey, 1983).

A nomenclature for multiple 5-HT receptors that is based on types of serotonergic binding sites in mammalian brain has been widely adapted. 5-HT_1 sites are labeled with high affinity by ^{3}H-5-HT and have relatively low affinity for many classical 5-HT antagonists and ketanserin (Peroutka and Snyder, 1979; Awouters et al., 1982). 5-HT_2 sites are labeled with high affinity by ^{3}H-spiperone and ^{3}H-ketanserin and have high affinity for many classical 5-HT antagonists (Peroutka and Snyder, 1979; Leysen et al., 1982). Whereas it has been shown that the 5-HT_2 sites correspond to certain functional 5-HT receptors (Peroutka et al., 1981; Van Nueten et al., 1981; Humphrey et al., 1982; Leysen et al., 1982; Awouters et al., 1982; Cohen et al., 1983; Maayani et al., 1984; Lucki et al., 1984), the functional significance of 5-HT_1 sites is still debated (Leysen and Tollenaere, 1982; Fozard, 1983b). Evaluating the significance of 5-HT_1 sites may be confounded by the fact that there are actually at least two different sites labeled with the same high affinity by ^{3}H-5-HT: 5-HT_{1A} sites are distinguished from

5-HT_{1B} sites by the relatively high affinity of the 5-HT_{1A} sites for the neuroleptic spiperone and the centrally active serotonergic agonist 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT) (Pedigo et al., 1981; Schnellmann et al., 1984; Middlemiss and Fozard, 1983; Hamon et al., 1984b).

5-HT receptors that are homologous to the 5-HT_2 binding site mediate platelet aggregation and contraction of certain vascular muscle and play a role in the rodent "head shake" response. 5-HT is competitively antagonized at 5-HT_2 receptors by ketanserin, spiperone, and classical 5-HT antagonists; the dissociation constants of these antagonists are in the low nanomolar range. Recent data suggest that the activation of 5-HT_2 receptors is associated with alterations in phosphatidylinositol metabolism (Kendall and Nahorski, 1984; de Chaffoy de Courcelles et al., 1984; Conn and Sanders-Bush, 1984) and an increase in intracellular free Ca^{++} (Affolter et al., 1984). Responses that appear to be mediated by receptors of the 5-HT_2 type are listed in Table 1.

A "peripheral neuronal" type of 5-HT receptor has been identified on sympathetic neurons of the rabbit heart and on vagal afferent fibers (Fozard and Mobarok Ali, 1978; Fozard et al., 1979; Fastier et al., 1959; Fortune at al., 1983; Fozard, 1983a; Fozard, 1984); this receptor may also mediate the pain-producing effect of 5-HT (Fastier et al., 1959; Donatsch et al., 1984). The peripheral neuronal receptor is relatively insensitive to classical 5-HT antagonists but is

Table 1. Responses that appear to be mediated by the 5-HT_2 receptor

System	References
VASCULAR CONTRACTION:	
1) rabbit aorta	Apperley et al., 1976 Purdy et al., 1981 Humphrey et al., 1982 Stollak & Furchgott, 1983 Bradley et al., 1983 Maayani et al., 1984
2) rat aorta	Cohen et al., 1981 Forster and Whalley, 1982 Bradley et al., 1983
3) rat jugular vein	Cohen et al., 1981, 1983
4) rat caudal artery	Van Nueten et al., 1981 Leysen et al., 1982 Bradley et al., 1983
5) rat portal vein	Lemberger et al., 1984
6) calf coronary artery	Kaumann, 1983b
7) dog femoral artery	Apperley et al., 1980 Black et al., 1981
8) sheep umbilical artery	Dyer and Gant, 1973 Dyer, 1983
9) some pulmonary blood vessels	Eyre, 1975 Chand and Altura, 1980
10) carotid artery (dog, rabbit, pig)	Apperley et al., 1980 Black et al., 1981 Verdouw et al., 1984
11) other extracranial arteries	Edvinsson et al., 1978 Lamar and Edvinsson, 1980 Apperley et al., 1980

Table 1. (continued)

System	References
NON-VASCULAR CONTRACTION:	
1) rat uterus	Gaddum and Hameed, 1954 Millar et al., 1982 Ichida et al., 1983 Cohen and Schenck, 1984
2) guinea pig trachea	Van Nueten et al., 1982 Cohen and Schenck, 1984
OTHER RESPONSES:	
1) platelet aggregation and shape change	Boullin et al., 1978 DeClerck et al., 1984 Affolter et al., 1984
2) prostacyclin synthesis	Coughlin et al., 1984
3) rodent "head shake" behavior	Peroutka et al., 1981 Colpaert and Janssen, 1983 Lucki et al., 1984
4) rat paw edema	Fozard & Middlemiss, 1983

blocked by (-)cocaine and a new class of tropanyl-ester antagonists that includes MDL 72222 and ICS 205-930 (Fozard et al., 1979; Fozard, 1984; G. Engel et al., 1984). 5-Methoxytryptamine is not an effective agonist at these receptors (Keele and Armstrong, 1964; Fozard and Mobarok Ali, 1978; Fozard, 1983a), in contrast with its potency at other types of 5-HT receptors (e.g. Vane, 1959; Fozard and Mobarok Ali, 1978; Engel et al., 1983; Clancy et al., 1983). The peripheral neuronal receptor, which was previously referred to as the 5-HT_{ETMIC} receptor (Wallis, 1981), is distinct from the 5-HT_1 and 5-HT_2 binding sites (Fozard, 1984; Engel et al., 1984). It has been suggested that the peripheral neuronal type 5-HT receptor be referred to as the 5-HT_3 receptor (Verdouw et al., 1984). Responses that appear to be mediated by receptors of the 5-HT_3 type are listed in Table 2.

Many 5-HT receptors, including those coupled to adenylate cyclase, remain incompletely characterized. Based on their relative insensitivity to ketanserin and/or classical antagonists, as well as the relative activity of certain serotonergic agonists, some "atypical" 5-HT receptors have been referred to as 5-HT_1-like (Peroutka et al., 1981; Engel et al., 1983; Lucki et al., 1984; Verdouw et al., 1984). The selectivity of the 5-carboxamido analog of 5-HT ($5\text{-CONH}_2\text{-T}$) may prove helpful in categorizing these 5-HT receptors for which a specific antagonist has not yet been described. Whereas $5\text{-CONH}_2\text{-T}$ is 30-fold less potent

Table 2. Responses that appear to be mediated by the "peripheral neuronal" ($5\text{-}HT_3$) receptor

System	References
1) excitation of sympathetic neurons of rabbit heart	Fozard & Mobarok Ali, 1978 Fozard et al., 1979 Fozard, 1984 G. Engel et al., 1984
2) excitation of vagal afferent fibers & Bezold-Jarisch reflex	Fastier et al., 1959 Ireland et al., 1983 Fortune et al., 1983 Fozard, 1983a, 1984 G. Engel et al., 1984
3) blister base pain	Fastier et al., 1959 Keele and Armstrong, 1964 Donatsch et al., 1984

than 5-HT in eliciting contraction through the 5-HT_2 receptor in rabbit aorta (Feniuk et al., 1981), it is significantly more potent than 5-HT at presynaptic autoreceptors in rat cortex (Engel et al., 1983), at pre- and post-synaptic 5-HT receptors in canine saphenous vein (Feniuk et al., 1981; Engel et al., 1983), at 5-HT receptors mediating relaxation of smooth muscle (Feniuk et al., 1984) and at ^{3}H-5-HT binding sites (Engel et al., 1983). 5-$CONH_2$-T seems to exhibit equally high affinity for both the 5-HT_{1A} and the 5-HT_{1B} site (Engel et al., 1983; Ebersole and Maayani, unpublished observations). 5-$CONH_2$-T is also extremely potent in elicting constriction of arteriovenous anastomoses and dilation of arterioles in the pig (Verdouw et al., 1984). The activity of 5-$CONH_2$-T on 5-HT receptors coupled to adenylate cyclase has not been reported. Unclassified 5-HT receptors, including those thought to correspond to 5-HT_1 binding sites, are listed in Table 3. It is possible that this group of "atypical" receptors will be shown to consist of several different classes of 5-HT receptors.

II. The Significance of "Atypical" 5-HT Receptors

Multiple receptors for a hormone or neurotransmitter are best defined by the identification of selective, competitve antagonists for each receptor class. Receptors that are relatively insensitive to known antagonists may be

Table 3. Responses mediated by "atypical" 5-HT receptors (* = 5-HT_1-like)

System	References
RESPONSES INVOLVING RELAXATION OF SMOOTH MUSCLE:	
1) guinea pig ileum*	Feniuk et al., 1983b, 1984
2) cat saphenous vein*	Feniuk et al., 1983b, 1984
3) pulmonary vein (sheep, goat)	Eyre, 1975 Chand, 1981
4) goat trachea	Chand et al., 1975
5) vasodilatation in guinea pig stomach	Van Nueten et al., 1983
6) porcine vena cava*	Trevethick et al., 1984
7) pre-synaptic receptor in dog saphenous vein*	Watts et al., 1981 Feniuk et al., 1979, 1981 Van Nueten et al., 1981 Humphrey et al., 1983 Engel et al., 1983
8) endothelium-dependent relaxation of coronary artery	Cocks and Angus, 1983
9) arterioles of porcine carotid bed*	Verdouw et al., 1984
OTHER RESPONSES:	
1) contraction of basilar artery* & other intracranial arteries	Edvinsson et al., 1978 Lamar and Edvinsson, 1980 Forster and Whalley, 1982 Peroutka et al., 1983 Cohen et al., 1984 (also see: Muller-Schweinitzer & Engel, 1983)
2) contraction of rat stomach fundus	Vane, 1959 Offermeier & Ariens, 1966 Awouters et al., 1982 Leysen et al., 1982 Wrigglesworth, 1983

Table 3. (continued)

System	References
3) post-synaptic receptor in dog saphenous vein*	Apperley et al., 1980 Feniuk et al., 1981, 1983a Engel et al., 1983
4) cat atrium (chronotropy and inotropy)	Kaumann, 1983a
5) constriction of arteriovenous anastomoses of porcine carotid bed*	Verdouw et al., 1984
6) excitation in symapthetic ganglia	Wallis, 1981
7) M-receptor of guinea pig ileum	Gaddum and Picarelli, 1957 Fozard & Mobarok Ali, 1978 Fozard et al., 1979 Costa and Furness, 1979 Awouters et al., 1982 Fozard, 1984
8) D-receptor of guinea pig ileum	Gaddum and Picarelli, 1957 Barlow and Khan, 1959 Fozard & Mobarok Ali, 1978 Costa and Furness, 1979 Awouters et al., 1982 Pfeuffer-Friedrich and Kilbinger, 1984
9) autoreceptor in rat brain* (inhibition of 5-HT release)	Baumann & Waldmeier, 1981 Martin & Sanders-Bush, 1983 Engel et al., 1983 Middlemiss, 1984a, 1984b
10) inhibitory electrophysiological responses in rat brain*	Aghajanian, 1981 Peroutka et al., 1981
11) glycogenolysis in mouse cortex	Quach et al., 1982
12) "serotonin behavioral syndrome" in rats*	Jacobs, 1974 Lucki et al., 1984 Tricklebank, 1984

Table 3. (continued)

System	References
13) guinea pig myoclonus*	Luscombe et al., 1984
14) rotational behavior in rats with unilateral raphe lesions*	Blackburn et al., 1984
15) stimulation of adenylate cyclase activity*	(reviewed in text)

referred to as "atypical". This designation is often transient: beta-adrenergic and histamine H_2 receptors were both considered "atypical" at one time. Systematic pharmacological characterization of these receptors in various tissue preparations (Ash and Schild, 1966; Black et al., 1972, 1982; Furchgott, 1972) yielded insights about receptor classification and led to the development of new therapeutic agents.

Responses mediated by "atypical" 5-HT receptors also have potential physiological and pathophysiological importance. As seen in Table 3, activation of many of these receptors leads to relaxation of vascular smooth muscle. It is possible that selective agonists for these receptors would be useful in the treatment of hypertension, coronary artery spasm, or migraine (Webb, 1981, Spierings and Saxena, 1982). A selective antagonist for the "atypical" 5-HT receptors that mediate contraction of intracranial arteries may be useful in the treatment of migraine, vasospasm, and cerebral ischemia (Peroutka and Kuhar, 1984).

Although 5-HT is thought to play a significant role in mammalian brain, many central 5-HT receptors remain incompletely characterized. Electrophysiological and biochemical studies have revealed what may be two types of 5-HT "autoreceptor". The 5-HT autoreceptor located on raphe cell bodies or dendrites mediates inhibition of neuronal firing rate (Aghajanian, 1981); the autoreceptor located on presynaptic terminals mediates inhibition of 5-HT release

(Baumann and Waldmeier, 1981; Martin and Sanders-Bush, 1982; Engel et al., 1983). Several central 5-HT receptors, including the autoreceptors, bear at least some resemblance to 5-HT_1 binding sites. 5-HT receptors whose similarities to 5-HT_1 sites have been noted include receptors that mediate the "serotonin behavioral syndrome", postsynaptic receptors that mediate inhibitory electrophysiological effects of 5-HT, and receptors linked to adenylate cyclase (Table 3).

III. Characterization of Cyclase-Linked 5-HT Receptors

A. Significance

Although the biochemical, physiological, and behavioral consequences of 5-HT-stimulated cAMP accumulation have been well-studied in invertebrate systems, relatively little is known about the pharmacology and physiological significance of mammalian 5-HT receptors coupled to adenylate cyclase. Adenylate cyclase systems, including those from brain, have proved to be just as useful as isolated organ preparations for the quantitative characterization of "physiologically relevant" receptors, and have been especially important in defining receptors in mammalian brain. Beta-adrenergic receptors (Kaumann and Birnbaumer, 1974; Dolphin et al., 1979) and histamine H_2 receptors (Green et al., 1977; Maayani et al., 1981), for example, have both been unambiguously characterized in broken-cell adenylate cyclase assays. The value of these assay systems is also

well-illustrated by the progress that has been made in elucidating the roles of multiple central and peripheral dopamine receptors. Most of the known actions of dopamine on neuronal, vascular, and endocrine systems now appear to be explained by dopamine receptors that either stimulate (D_1 receptors) or inhibit (D_2 receptors) adenylate cyclase activity (Kebabian and Calne, 1979; Enjalbert and Bockaert, 1983). Furthermore, dopamine-sensitive adenylate cyclase systems have proved to be excellent model systems for studying structure-activity relationships of dopaminergic agonists and antagonists (e.g., Iverson, 1975; Itoh et al., 1984).

B. Background

5-HT causes a concentration-dependent increase in cAMP accumulation that is apparently receptor-mediated in many invertebrate and mammalian preparations. Half-maximal stimulation is usually produced by 0.1 to 2 uM 5-HT. Invertebrate systems where 5-HT receptors coupled to adenylate cyclase have been described include cockroach thoracic ganglion (Nathanson and Greengard, 1974), mollusc heart (Higgins, 1977; Kebabian et al., 1979), mollusc gill (Kebabian et al., 1979; Weiss and Drummond, 1981), mollusc nervous tissue (Cedar and Schwartz, 1972), liver fluke (Northup and Mansour, 1978; McNall and Mansour, 1984), planaria (Franquinet et al., 1978), snail neurons (Drummond et al., 1980; Deterre et al., 1982), earthworm nervous

tissue (Robertson and Osborne, 1979), leech muscle and ganglia (Biondi et al., 1982), silkmoth brain (Rasenick and Berry, 1981), and blowfly salivary gland (Berridge and Heslop, 1981). In cell-free preparations of cockroach ganglion and liver fluke the action of 2-bromo-LSD was carefully evaluated: it appears to be a potent competitve antagonist of 5-HT receptors in those systems, with dissociation constants of 5 nM and 8 nM, respectively. These values are higher than the dissociation constant of 2-bromo-LSD for the 5-HT_2 receptor, 0.2 nM (Stollak and Furchgott, 1983); the relatively low potencies of other classical antagonists that were tested (Nathanson and Greengard, 1974; McNall and Mansour, 1984) indicate that the 5-HT receptors coupled to cyclase in liver fluke and cockroach are not 5-HT_2-like.

5-HT elicits increases in cAMP production in a variety of peripheral mammalian tissues and cultured cell systems: rat skeletal muscle (Garber, 1977; Ezrailson et al., 1983), rat kidney (Shah et al., 1979), porcine vena cava (Trevethick et al., 1984), rabbit cornea (Neufeld et al., 1982), cultured calf aorta smooth muscle cells (Luchins and Makman, 1980), NCB-20 neuroblastoma-brain hybrid cells (MacDermot et al., 1979; Berry-Kravis and Dawson, 1983), and growth-inhibited rat fibroblasts (Hauser, 1982). Where they were tested, classical 5-HT antagonists did not appear to be extremely potent.

In mammalian brain, a 5-HT-dependent increase in cAMP accumulation was first observed in slices of rabbit cerebellum (Kakiuchi and Rall, 1968). The effect was subsequently described in slices of cerebral cortex from guinea pig (Huang et al., 1971; Huang and Daly, 1972), squirrel monkey (Skolnick et al., 1973), and humans (Kodama et al., 1973; Tsang and Lal, 1977) and in slices of rat hypothalamus (Daszuta et al., 1979) and rat hippocampus (Fuxe et al., 1983). Pharmacological characterization of the 5-HT receptors that may mediate those responses has not been performed.

Von Hungen and co-workers were the first to report 5-HT-stimulated adenylate cyclase activity in a cell-free preparation of mammalian brain (Von Hungen and Roberts, 1973; Von Hungen et al., 1974, 1975); they also made the important observation that percent stimulation of cAMP production by 5-HT decreases markedly during rat maturation, a finding that was confirmed by others (Enjalbert et al., 1978a; Daszuta et al., 1979). This decrease in percent stimulation is attributable to the fact that 5-HT-stimulated enzyme activity does not increase in proportion to basal activity during development. 5-HT produces a marked stimulation of adenylate cyclase activity (80-140% above basal; EC_{50} = 1 uM) in homogenates of several structures from newborn rat CNS, including colliculi, hippocampus, hypothalamus, and lumbar spinal cord (Von Hungen et al., 1974, 1975; Enjalbert et al., 1978a; Mallat and Hamon,

1982). 5-HT-stimulated activity is also found in hypothalamic homogenates of fetal guinea pig (Enjalbert et al., 1978a) and in dissociated and cultured cells derived from embryonic or newborn mouse brain (Ebersolt et al., 1981a; Premont et al., 1983; Chneiweiss et al., 1984).

Because most studies of the functions associated with central 5-HT receptors have been done in mature animals, however, investigators have continued to seek measurable 5-HT-stimulated adenylate cyclase activity in cell-free preparations of adult brain. 5-HT-stimulated activity (40-100%) has been described in membrane preparations from several species: monkey anterior limbic cortex (Ahn and Makman, 1978a,b), rabbit frontal cortex and anterior limbic cortex (Rosenfeld and Makman, 1981), horse striatum (Fillion et al., 1977, 1979b, 1980a), rat whole brain (Pagel et al., 1976), rat preoptic area (Jansco and Wollemann, 1977), rat striatum (Fillion et al., 1979a), rat hippocampus (Barbaccia et al., 1982, 1983; Shenker et al., 1983b), and guinea pig hypothalamus (Ahn and Makman, 1977). Publications based on results from this thesis (Shenker et al., 1982, 1983a, 1983b) have described 5-HT-stimulated activity in membranes of adult guinea pig hippocampus.

Experiments in which 5-HT-stimulated adenylate cyclase activity was measured in membranes prepared from horse or rat striatum, or from cultured cells, led Fillion and co-workers to the conclusion that there are two distinct types of 5-HT-stimulated adenylate cyclase activity in

mammalian brain (reviewed by Fillion et al., 1983). One type (EC50 = 1 uM) seems to correspond to the stimulation routinely reported in rodent brain; Fillion and co-workers suggest that it is located on glial membranes. The other type is a "high affinity" stimulation (EC50 = 1 nM) which is thought to be localized on postsynaptic neuronal membranes. Other investigators were unable to detect significant stimulation of adenylate cyclase activity by nanomolar concentrations of 5-HT (Nelson et al., 1980a; Hamon et al., 1980; Barbaccia et al., 1983). Furthermore, most of the evidence from lesion studies (Enjalbert et al., 1978a; Nelson et al., 1980b; Barbaccia et al., 1983; Hamon et al., 1981) and from experiments with cultured brain cells (Ebersolt et al., 1981a; Premont et al., 1983; Chneiweiss et al., 1984) suggests that that rodent brain cyclase systems that are stimulated by micromolar concentrations of 5-HT are localized on postsynaptic neurons, not glia.

Although stimulation of cAMP accumulation by 5-HT has been reported in many preparations from mammalian brain, only with the infant rat colliculi system were attempts made to conduct the range of pharmacological tests needed to characterize receptors (Enjalbert et al., 1978a,b). The ergot alkaloid metergoline was the only drug found to antagonize 5-HT in a competitive manner, with an apparent dissociation constant of 1 uM. Other classical 5-HT antagonists (e.g., cyproheptadine, methiothepin, cinanserin) produced a relatively weak, non-surmountable inhibition of

the 5-HT response. The IC50 value of spiperone (to inhibit the response to 10 uM 5-HT) was reported to be 4 - 12 uM (Nelson et al., 1979, 1980a). The activities of only a few agonists other than 5-HT have been described.

C. Desensitization

Decreased responsiveness to 5-HT over time has been reported in cell-free adenylate cyclase systems from monkey anterior limbic cortex (Ahn and Makman, 1978a), snail CNS (Drummond et al., 1980), horse striatum (Fillion et al., 1981), and liver fluke (McNall and Mansour, 1984b). In the snail system, the decrease in responsiveness was also shown to occur during preincubation in the absence of added 5-HT, indicating that the "deterioration" of 5-HT receptors, rather than desensitization, was responsible.

Most studies on "supersensitivity" and "subsensitivity" of central monoamine receptors have focused on the effects of chronic impairment or enhancement of neurotransmission. Up- and down-regulation of serotonergic binding sites in brain occur in response to various treatments (reviewed by Hamon et al., 1984a). Altered sensitivity of brain 5-HT receptors may also be found in humans with affective disorders (e.g., Meltzer et al., 1984; Siever et al., 1984). Rapid and specific desensitization of cyclase-linked brain receptors for isoproterenol (Wagner and Davis, 1979) and dopamine (Memo et al., 1982) has been studied in vitro. Quantitative study of desensitization of central 5-HT

receptors coupled to adenylate cyclase is an important first step in understanding whether rapid regulation of these receptors is a normal physiological phenomenon, or whether it might become significant during pharmacological therapy or pathophysiological processes.

D. Relation to 5-HT_1 Binding Sites

Whether 5-HT receptors coupled to adenylate cyclase are related to 5-HT_1 binding sites in brain is controversial. Peroutka and co-workers (1981) showed a correlation between the pharmacological properties of 5-HT_1 sites and those of 5-HT receptors linked to cyclase, however the methods used to construct the correlation have been questioned (Middlemiss, 1982; Hamon et al., 1984a). Fillion and co-workers (1980b, 1981) have suggested that the 5-HT_1 site represents an "uncoupled" or "inactivated" form of a neuronal cyclase-linked 5-HT receptor, exhibiting increased affinity for agonists. The cyclase-coupled 5-HT receptors in adult rat hippocampus (Barbaccia et al., 1983) and in cultured NCB-20 cells (Berry-Kravis and Dawson, 1983) both appear 5-HT_1-like. The most complete study of the relationship between cyclase-coupled 5-HT receptors and 5-HT_1 binding sites was performed in infant rat brain by Nelson and co-workers (1980a, 1980b). Properties of the cyclase-linked 5-HT receptors in infant rat brain, especially those in colliculi, indicate that they are not homologous to 5-HT_1 binding sites. The evidence includes

the finding that several serotonergic agonists with relatively high affinity for ^{3}H-5-HT binding sites (e.g., m-trifluoromethylphenylpiperazine (TFMPP) and 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl) 1H indole (RU 24969)) have apparently no affinity for 5-HT receptors coupled to adenylate cyclase in infant rat colliculi (Nelson et al., 1980a; Euvard and Boissier, 1980).

In interpreting these conflicting results on the relationship between cyclase-linked 5-HT receptors and 5-HT_1 binding sites, it is important to consider the relatively recent finding that 5-HT_1 sites are not homogeneous. The IC50 value of a compound in competing for a mixture of binding sites labeled by ^{3}H-5-HT has limited meaning (Schnellmann et al., 1984). Furthermore, it has been suggested that there is more than one type of 5-HT receptor positively coupled to adenylate cyclase (Von Hungen et al., 1975; Fillion et al., 1979; Peroutka et al., 1981), as is seen with the cyclase-coupled $beta_1$- and $beta_2$-adrenergic receptors (e.g., Minneman et al., 1979; Dolphin et al., 1979; Hedberg and Mattsson, 1981; Ebersolt et al., 1981b).

IV. Aims of This Study

Study of 5-HT-stimulated adenylate cyclase activity in adult guinea pig hippocampal membranes was undertaken with the following aims:

1) Develop experimental conditions that provide enhanced stimulation of adenylate cyclase activity by 5-HT

Maximal stimulation of adenylate cyclase activity by 5-HT in brain membranes from common adult laboratory animals is often no greater than 50% (Ahn and Makman, 1977; Enjalbert et al., 1978a; Fillion, 1983; Barbaccia et al., 1983). Although receptor-mediated stimulation of adenylate cyclase activity in brain membranes may not be as high as that found in peripheral systems, methodological improvements have allowed neurotransmitter receptors to be studied in detail (Bockaert, 1981). These improvements have included modification of assay conditions and the use of microdiscs of brain tissue that contain a high density of receptors.

Enhanced stimulation by 5-HT was sought by altering the assay conditions used to measure histamine-stimulated adenylate cyclase activity in guinea pig hippocampal membranes. The histamine H_2 receptor which mediates this response has been well-characterized (Hegstrand et al., 1976; Green et al., 1977; Maayani et al., 1981). Although most studies of of mammalian serotonergic systems are done with rats, preliminary experiments indicated that the

maximal percent stimulation by 5-HT in rat hippocampus was much less than that in guinea pig hippocampus.

The possibility was considered that 5-HT receptors linked to cyclase in guinea pig hippocampus undergo desensitization, as had been shown for many other 5-HT receptors. To explore the hypothesis that exposure to 5-HT *in vivo* contributed to the low responsiveness of the cyclase system seen *in vitro*, guinea pigs were treated with reserpine to deplete brain 5-HT. Desensitization of the cyclase-coupled 5-HT receptors was also studied *in vitro*.

2) Establish that response to 5-HT is mediated by 5-HT receptors

The dependency of the response to 5-HT on GTP was evaluated because dependency on guanine nucleotides is a property of receptor-mediated increases in adenylate cyclase activity (see Birnbaumer and Iyengar, 1982).

5-HT and serotonergic agonists have been shown to cause effects by activating receptors for other biogenic amines directly, as discussed earlier, or indirectly, by releasing endogenous catecholamines (Benfey et al., 1974; Humphrey, 1978). Lack of simple additivity of 5-HT-stimulated and dopamine-stimulated adenylate cyclase activites (Enjalbert et al., 1978b; Weiss and Drummond, 1981) may be due to activity of 5-HT at dopamine receptors. Beta-adrenergic and dopamine receptors coupled to adenylate cyclase have been found in mammalian hippocampus (Dolphin et al., 1979;

Barbaccia et al., 1983). To exclude the possibility that some or all of the response to 5-HT in guinea pig hippocampal membranes was due to activation of catecholamine receptors, stimulation by the beta-adrenergic agonist (-)isoproterenol and by dopamine was investigated.

3) Characterize the receptors with agonists and antagonists

Research on classification of multiple 5-HT receptors has been revitalized by the description of new serotonergic agonists and antagonists. The drugs used in this study were chosen on the basis of their defined effects on other 5-HT receptors. The dissociation constants of antagonists for the 5-HT_2 receptor (ketanserin, spiperone) and for the 5-HT_3 receptor ((-)cocaine, MDL 72222) have been determined. In addition to its high affinity for 5-HT_2 receptors, spiperone clearly discriminates between 5-HT_{1A} and 5-HT_{1B} binding sites in brain, and was described as a weak inhibitor of 5-HT-stimulated cyclase activity in infant rat colliculi. $5\text{-CONH}_2\text{-T}$ was used because it appears to be a selective agonist for several of the "atypical" 5-HT receptors.

Two novel compounds whose actions on central serotonergic receptors have recently been described, 8-OH-DPAT and buspirone, were also used. The potent agonist actions of 8-OH-DPAT include inhibition of firing of raphe serotonergic neurons and production of the serotonin behavioral syndrome (Hjorth et al., 1982; Fallon et al.,

1983; Tricklebank, 1984). 8-OH-DPAT is the only compound other than spiperone reported to have high selectivity for the 5-HT_{1A} binding site; 8-OH-DPAT has relatively poor affinity for the 5-HT_2 binding site, for the cyclase-linked 5-HT receptor in infant rat colliculi, and for the 5-HT autoreceptor in cortex (Middlemiss and Fozard, 1983; Hamon et al., 1984b; Middlemiss, 1984a). ^{3}H-8-OH-DPAT has been used to label the 5-HT_{1A} site in brain (Gozlan et al., 1983; Marcinkiewicz et al., 1984).

Buspirone is an atypical, anxioselective anxiolytic with promising clinical applications and an unknown mechanism of action (Taylor et al., 1984). Attention has recently focused on the agonist effects of buspirone on serotonergic systems in mammalian brain (Hjorth and Carlsson, 1982). Buspirone potently inhibits the firing of raphe serotonergic neurons (VanderMaelen and Wilderman, 1984) and elicits at least some features of the serotonin behavioral syndrome (Hjorth and Carlsson, 1982; Eison et al., 1983). Although buspirone was originally described as "inactive" in competing for 5-HT_1 binding sites (Stanton et al., 1981), other data suggest that it has relatively high affinity for the 5-HT_{1A} site in hippocampus (Glaser and Traber, 1983; Gozlan et al., 1983).

Studies were performed with the intention of comparing the pharmacological properties of the cyclase-linked 5-HT receptors in guinea pig hippocampus to the known pharmacological properties of: 5-HT_2 receptors, 5-HT_3

receptors, cyclase-linked 5-HT receptors in infant rat colliculi, other "atypical" 5-HT receptors in the brain and periphery, and 5-HT_{1A} and 5-HT_{1B} binding sites.

METHODS AND MATERIALS

The methods used to conduct and analyze a routine adenylate cyclase assay are described below. Earlier versions of the assay are discussed in the Results section.

I. Membrane Preparation

Male Hartley-Albino guinea pigs (400-450 g) (Perfection Breeders, Douglassville, PA) were killed by decapitation and the hippocampi of each animal were dissected out. Ice-cold tissue buffer (TB) (pH 7.4 at 23°) containing 300 mM sucrose, 20 mM Tris HCl, 1 mM EGTA, 5 mM Na_2EDTA, and 5 mM dithiothreitol was prepared fresh daily. The hippocampi from each animal were homogenized by hand in 9 ml TB (20 strokes, Arthur H. Thomas size C Teflon pestle tissue homogenizer); in some experiments, each hippocampus was homogenized separately in 4.5 ml TB. The homogenate was diluted 1:8 with TB and centrifuged at 39,000 x g for 10 min at 4°C. Pellets were resuspended by vortexing in the same volume used for homogenization. This particulate fraction was stored on ice until used within the hour.

II. Adenylate Cyclase Assay

Adenylate cyclase activity was determined by measuring the conversion of α-^{32}P-ATP to ^{32}P-cAMP. Assay components (200 ul) were first incubated for 5 min at 30°C. The reaction was initiated with 50 ul of the hippocampal preparation, yielding the following final assay mixture:

80 mM Tris HCl (pH 7.4), 0.2 mM ATP, 2 mM Mg acetate, 10 or 20 uM GTP, 10 uM pargyline, 0.6 mM ascorbate, 4 mM theophylline, 1 mM cAMP, 125 ug creatine phosphokinase, 5 mM creatine phosphate, 1.5 uCi α-^{32}P-ATP (10-50 Ci/mmol), 100 ug particulate protein, 60 mM sucrose, 0.2 mM EGTA, 1 mM Na_2EDTA, 1 mM dithiothreitol, and various concentrations of drugs. The incubation was carried out at 30°C for 2 min; under these conditions, enzyme activity was linear with respect to time and protein concentration. To confirm that these conditions were adequate for drug-receptor equilibration, certain experiments were designed differently: the hippocampal membranes were first incubated with drugs and assay components (lacking α-^{32}P-ATP) for 8 min at 30°C; the assay was then initiated with α-^{32}P-ATP and conducted for the usual 2 min. Assays were stopped by the addition of 100 ul of a solution containing 2% sodium lauryl sulfate, 45 mM ATP, and Tris, pH 7.5. After addition of ^{3}H-cAMP (10,000-30,000 CPM) to monitor recovery, the samples were boiled for 3 min, cooled to room temperature, and ^{32}P-cAMP was isolated exactly as described by Salomon (1979). Recovery averaged about 70%, and reaction blanks usually represented only 5% of the lowest measured enzyme activity. Protein was determined by the method of Lowry (Lowry et al., 1951), with bovine serum albumin as the standard and TB as the blank.

III. Data Analysis

Determinations were done at least in triplicate; the coefficient of variation was routinely $\leq$ 5%. Adenylate cyclase activity was expressed as pmoles cAMP/min/mg protein. Stimulation of adenylate cyclase activity was calculated by subtracting mean enzyme activity in the absence of agonist (basal activity) from the mean activity in the presence of agonist. The standard error of the net stimulation was calculated according to the formula provided by Hinkle et al. (1979). Percent stimulation was defined as (stimulation/basal) X 100. Concentration- response data were initially fit to a form of the logistic function (Parker and Waud, 1971; Johnson, 1982):

$$E = Emax/[1 + (EC50/[A])^N]$$

where E = response to agonist, Emax = maximal response to agonist, EC50 = concentration of agonist eliciting half-maximal response, [A] = agonist concentration, and N = slope index (a parameter that describes the steepness of the curve).

Additional analysis was performed with the computerized, nonlinear least-squares curve-fitting procedure FITFUN (Baig and Reid-Miller, 1980). Weighting was not used because the response variable (increase in adenylate cyclase activity) exhibits uniformity of variance (Johnson, 1982; unpublished observations). Curves from the

same experiment were often fit simultaneously; the statistical advantages of this method have been discussed (DeLean et al., 1978). More information on the models used to fit the data is presented in the Results section.

A partial F-test (see DeLean et al., 1978; Burgisser, 1983) was used to determine if a more complex model provided a significantly better fit to the data than a simple one. For example, the parallelism of concentration-response curves in the absence and presence of antagonist was tested by simulataneously fitting the set of curves to logistic functions where the slope indices were either allowed to vary ("variable slope model") or constrained to the same value ("common slope model") (Waud and Parker, 1971; Johnson, 1982). If the more complex variable slope model provided a significantly better fit to the data, it was inferred that the curves were not parallel.

The natural logs of the fitted estimates of EC50 values and dissociation constants were used to calculate geometric means $\pm$ SE (Fleming et al., 1972; DeLean et al., 1982). All other data are expressed as arithmetic means $\pm$ SE.

Differences were tested for statistical significance with Student's t-test; mean agonist slope indices were compared with one-way analysis of variance followed by Dunnett's test. Mean differences between parameters from control and reserpinized animals were tested for statistical significance by Student's t-test for paired data. For all statistical tests, the significance level was set at 0.05.

The dissociation constant of spiperone was determined from a Schild plot (Arunlakshana and Schild, 1959; Tallarida et al., 1979). If the slope of the plot was not significantly different from 1.0, the intercept of a line constrained to slope = 1.0 was used (Waud and Parker, 1971; Tallarida et al., 1979).

Data analysis was done on the PROPHET computer system, a national resource supported by the Chemical-Biological Information Handling Program, Division of Research Resources, National Institutes of Health.

IV. Reserpine Treatment

A fresh solution of reserpine was prepared for each experiment by dissolving 25 mg in 125 ul glacial acetic acid and 250 ul propylene glycol and diluting the mixture to 5 ml with distilled water. In each of four experiments, a guinea pig was injected i.p. with 5 mg reserpine/kg body weight, 25-27 hours before it was killed. This protocol is known to deplete 5-HT in guinea pig brain (Waalkes et al., 1959). At the same time that an animal was injected with reserpine, a paired control animal was injected with vehicle; in one experiment, the control animal was untreated. No differences were noted between preparations from untreated and vehicle-injected animals. All reserpinized animals exhibited at least 10% weight loss, ptosis, and huddled posture. Hippocampal membranes from each pair of animals

were prepared and assayed concurrently with the same set of reagents.

V. MATERIALS

The following drugs were generously donated: ketanserin tartrate and spiperone (Janssen Pharmaceutica, Beerse, Belgium); MDL 72222 methanesulphonate (Centre de Recherche Merrell International, Strasbourg, France); 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT) HBr (Lilly Research Laboratories, Indianapolis, Indiana); 5-$CONH_2$-T fumarate (Sandoz Ltd., Basle, Switzerland); (-)cocaine HCl, bufotenine (National Institute on Drug Abuse); buspirone HCl (Bristol-Myers, Evansville, Indiana).

Compounds obtained from commercial sources were: 5-HT HCl, 5-MeOT HCl, tryptamine HCl, reserpine (Sigma Chemical Co.), and bufotenine (Aldrich Chemical Co.). Radiochemicals were from New England Nuclear. Other reagents were obtained commercially and were of reagent grade purity.

RESULTS

I. Assay Modifications

Adenylate cyclase activity was initially measured in a crude hippocampal homogenate prepared in ice-cold bufffer composed of 0.32 M sucrose, 1 mM EGTA, and 5 mM Tris HCl, pH = 7.4 at 23°. The original assay mixture included 1 mM unlabeled ATP, 3 uCi α-^{32}P-ATP, and 5 mM Mg^{++}, but did not contain EDTA or DTT. The original mixture was otherwise like the final standard mixture described in Methods. Because preliminary experiments indicated that percent stimulation by 5-HT declined with time, a two minute assay was used. Under these original conditions (n = 14), basal adenylate cyclase activity was 151 $\pm$ 5 pmol cAMP/min/mg protein. Stimulation by 5-HT was concentration-dependent, with a slope index of approximately 0.7 and an EC50 of 0.1 uM. Maximal stimulation by 5-HT (10 uM) averaged 46 $\pm$ 2% over basal activity. Maximal stimulation by histamine averaged 102 $\pm$ 3%. The following sequence of changes led to assay conditions that provided enhanced stimulation of adenylate cyclase activity by 5-HT, as well as by histamine.

(1) The particulate preparation of hippocampus was first used to study the GTP-dependence of the 5-HT response without the interference of endogenous guanine nucleotides. The particulate preparation yielded about the same percent

stimulation by 5-HT as the homogenate, and it was subsequently adapted for routine use.

(2) Enhanced 5-HT-stimulated adenylate cyclase activity in planaria membranes has been reported as a result of including the metal chelator EDTA and the thiol-reducing agent 2-mercaptoethanol in the homogenization buffer (Franquinet et al., 1978). EDTA and a thiol-reducing agent (DTT) were also included in the buffer used to prepare liver fluke membranes for measurement of 5-HT-stimulated adenylate cyclase activity (Northup and Mansour, 1978).

The percent stimulation by 5-HT obtained in hippocampal membranes prepared in buffer containing 5 mM DTT and 5 mM EDTA (79 $\pm$ 5%) was significantly higher than that obtained in membranes prepared in the usual buffer (51 $\pm$ 1%) ($n = 3$, paired t-test). It was unclear whether this improvement was due to a decrease in basal activity, an increase in net stimulation, or both. Preliminary data suggested that buffer containing both 5 mM DTT and 5 mM EDTA provided higher percent stimulation by 5-HT than buffers containing either DTT or EDTA alone; tissue buffer containing both DTT and EDTA was used in all subsequent experiments.

(3) On the basis of two experiments in which a decrease in the added Mg^{++} concentration from 5 mM to 2 mM resulted in increased percent stimulation by 5-HT (68% vs 40%; 116% vs 83%), 2 mM added Mg^{++} was used in all subsequent work. To help compensate for the decrease in basal enzyme activity

that resulted from the lower free Mg^{++} concentration, the concentration of unlabeled ATP in the assay was decreased from 1 mM to 0.2 mM. This change increased the specific activity of the ^{32}P-ATP and did not appear to affect the percent stimulation by 5-HT.

The assay conditions that were adapted for routine use provided greater stimulation by 5-HT (maximal stimulation about 100% over basal activity) than the earlier conditions, although the slope index and the EC50 of the 5-HT concentration-response curve appeared to be the same. The improved assay conditions also provided enhanced maximal stimulation of adenylate cyclase activity by histamine (150%); other properties of the response to histamine (EC50, slope index, sensitivity to antagonists) appeared unchanged.

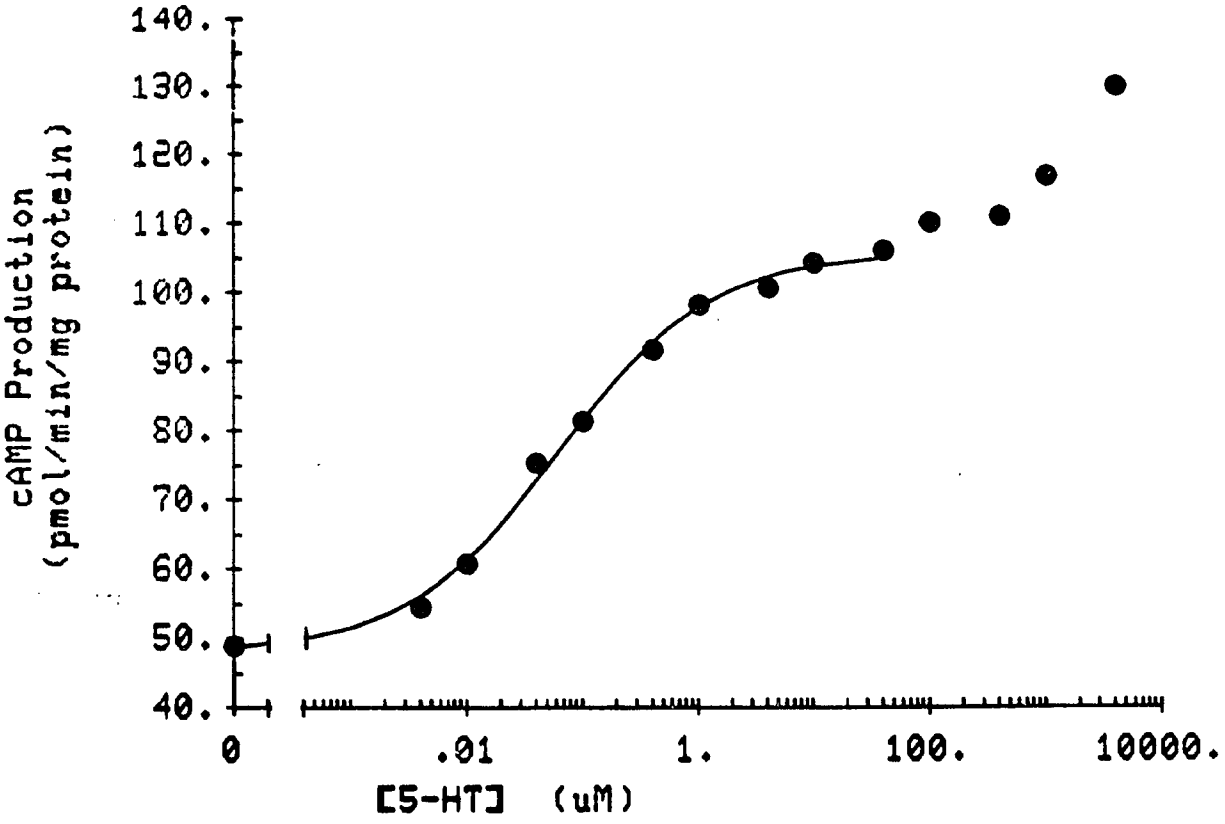
II. General Properties of the Response to 5-HT

A. Concentration-Response Curve

Basal adenylate cyclase activity averaged 49.4 $\pm$ 1.4 pmol cAMP/min/mg protein (n = 60). Stimulation of adenylate cyclase activity by 5-HT was concentration-dependent (Fig. 1). The stimulation reached a plateau at a concentration of about 10 uM 5-HT. Concentrations of 5-HT greater than 100 uM elicited an additional, non-saturable phase of stimulation (Fig. 1); other amines, including histamine, tryptamine, and amitriptyline, elicit similar stimulation

Fig. 1 Stimulation of adenylate cyclase activity by 5-HT

Data are from a single experiment. Responses to concentrations of 5-HT from 4 nM to 40 uM were fit to the logistic equation. The fit provided the following parameter estimates: EC50 = 60 nM, maximal response = 57.2 pmol cAMP/min/mg protein, slope index = 0.66.


cAMP Production
(pmol/min/mg protein)
140.
130.
120.
110.
100.
90.
80.
70.
60.
50.
40.
0
.01
1.
100.
10000.
[5-HT] (uM)

when used at very high concentrations (Trist, 1982; Maayani and Shenker, unpublished observations). Preliminary experiments indicated that the additional phase of stimulation elicited by 5-HT was unaffected by 5-HT antagonists, reinforcing the conclusion that it represents some type of "nonspecific" stimulation of the enzyme. In all additional work, only concentrations of 5-HT $\leq$ 100 uM were used.

Maximal stimulation by 5-HT averaged 104 $\pm$ 3% over basal activity (n = 33). The EC50 of 5-HT was 140 $\pm$ 11 nM. Concentration-response curves were shallow, with a mean slope index, 0.81 $\pm$ 0.02, that was significantly less than 1.0.

B. GTP-Dependence

The degree of stimulation elicited by a maximal concentration of 5-HT (10 uM) was dependent on exogenous GTP (Table 4). The maximal effect was observed with a GTP concentration of 10 uM. The small amount of stimulation seen in the absence of added GTP may be due to traces of endogenous guanine nucleotides carried into the assay with the membrane preparation.

C. Involvement of Receptors for Other Monoamines

Histamine-stimulated adenylate cyclase activity in guinea pig hippocampal membranes is mediated by histamine H_2 receptors (Hegstrand et al., 1976; Green et al., 1977; Maayani et al., 1981). Under the present assay conditions,

Table 4. GTP-dependence of stimulation by 5-HT

GTP (uM)	ADENYLATE CYCLASE ACTIVITY (pmoles cAMP/min/mg protein) Basal	+10 uM 5-HT	STIMULATION (%)
0	22.8 ± 0.6	26.0 ± 0.4	14
1	38.0 ± 0.9	53.8 ± 0.9	42
10	48.3 ± 1.2	95.1 ± 1.2	97
100	48.4 ± 0.7	95.4 ± 2.6	97

Each value is the mean ± SEM of quadruplicate determinations from one representative experiment.

maximal stimulation by histamine averaged 156 ± 2% over basal activity (n = 60). Maximally effective concentrations of 5-HT (10 uM) and histamine (400 uM) in combination produced additive stimulation (Fig. 2).

Stimulation of adenylate cyclase activity by the beta-adrenergic agonist (-)isoproterenol was concentration-dependent (Fig. 3), with a mean EC50 of 2 ± 1 uM (n = 3). Maximal stimulation by (-)isoproterenol averaged 31 ± 6% over basal activity, and was simply additive to the maximal stimulation by 5-HT (n = 4; Fig. 3), and to the maximal stimulation by histamine (n = 3).

Stimulation of adenylate cyclase activity by dopamine was also concentration-dependent (Fig. 4), with a mean EC50 of 15 ± 2 uM (n = 3). The stimulation elicited by 100 uM dopamine averaged 44 ± 4% over basal activity (n = 6). The stimulation elicited by 100 uM dopamine was simply additive to the maximal stimulation by histamine (n = 3). In contrast, the stimulation produced by a combination of 100 uM dopamine and a maximally effective concentration of 5-HT was significantly less than additive (n = 6; Fig. 4). Simple additivity of responses was observed when a lower concentration of dopamine (10 uM) was used (n = 3; Fig. 4). These findings suggest that stimulation by low concentrations of dopamine is mediated by receptors distinct from those for 5-HT (possibly D_1 dopamine receptors); part of the stimulation elicited by dopamine concentrations

Fig. 2 Additivity of responses to different agonists

Stimulation of adenylate cyclase activity by maximally effective concentrations of 5-HT (10 uM), tryptamine (100 uM), 5-MeOT (10 uM), and histamine (400 uM) was measured for each agonist alone and in combinations. Each bar represents the mean increase in cAMP production $\pm$ SE, from one representative experiment. Basal activity was 49.0 $\pm$ 0.8 pmol cAMP/min/mg protein.

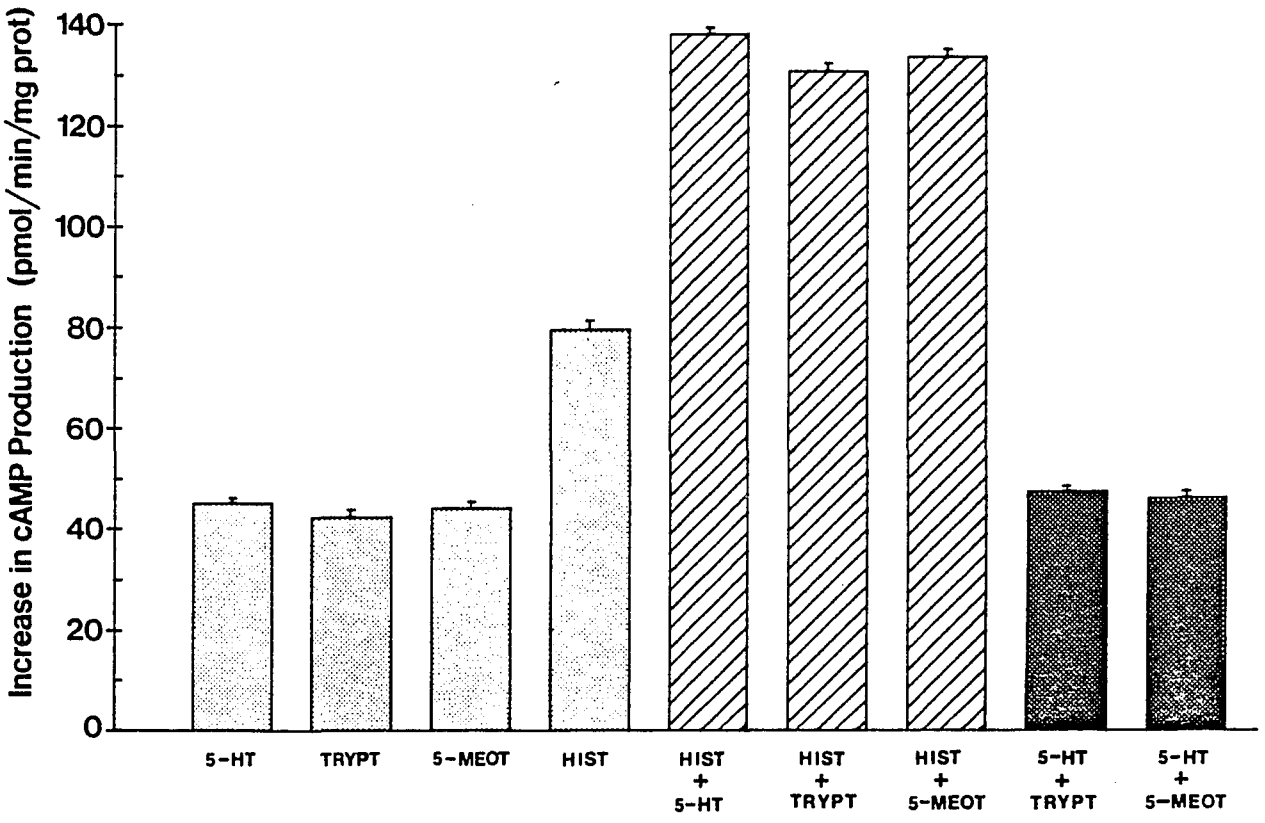

Increase in cAMP Production (pmol/min/mg prot)
140
120
100
80
60
40
20
0
5-HT
TRYPT
5-MEOT
HIST
HIST + 5-HT
HIST + TRYPT
HIST + 5-MEOT
5-HT + TRYPT
5-HT + 5-MEOT

Fig. 3 (-)Isoproterenol-stimulated adenylate cyclase activity

Stimulation of adenylate cyclase activity by 5-HT (●) and by (-)isoproterenol (■). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 66.0 ± 0.9 pmol cAMP/min/mg protein. The curves are best fits to the logistic function: for 5-HT, EC50 = 135 nM, maximal response = 57.9 pmol cAMP/min/mg protein, slope index = 0.89; for (-)isoproterenol, EC50 = 1.1 uM, maximal response = 23.1 pmol cAMP/min/mg protein, slope index = 1.09. The bar represents the mean increase in cAMP production (± SE) produced by a combination of 10 uM 5-HT and 40 uM (-)isoproterenol. The responses are simply additive.

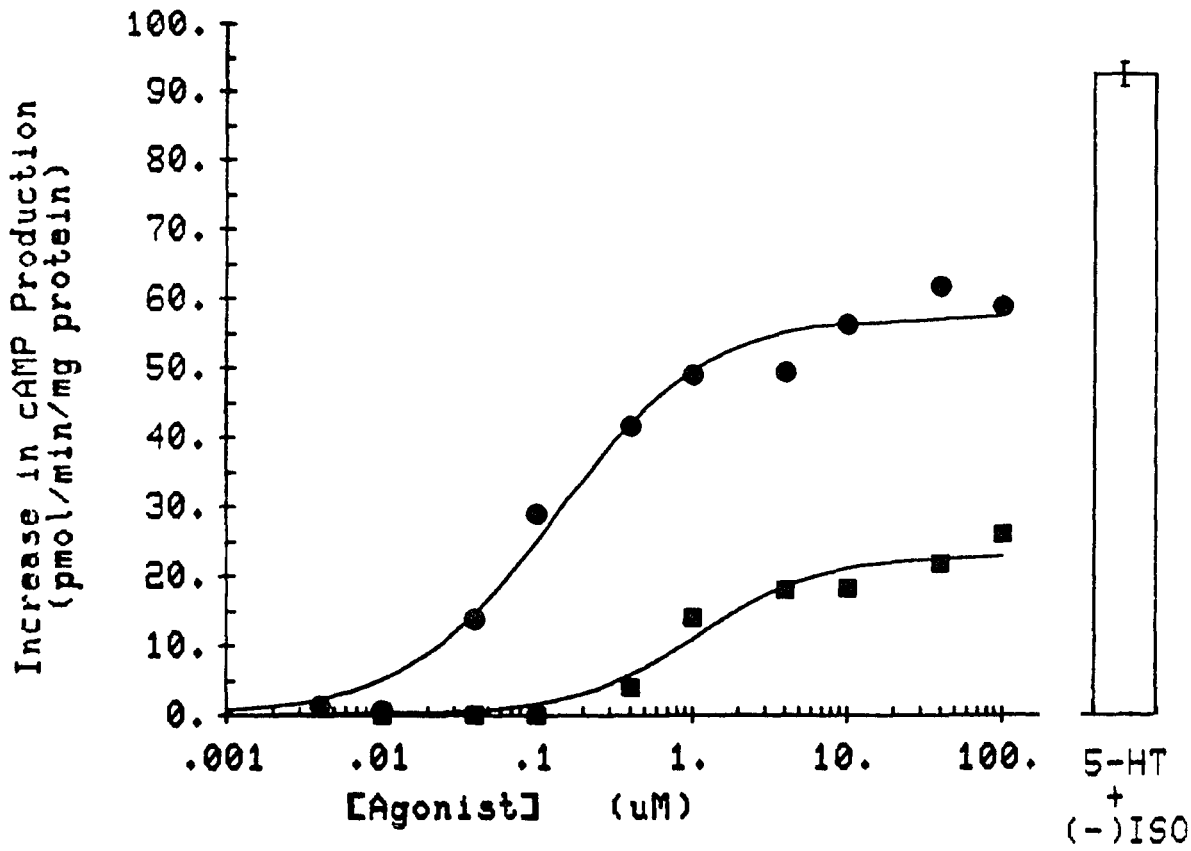
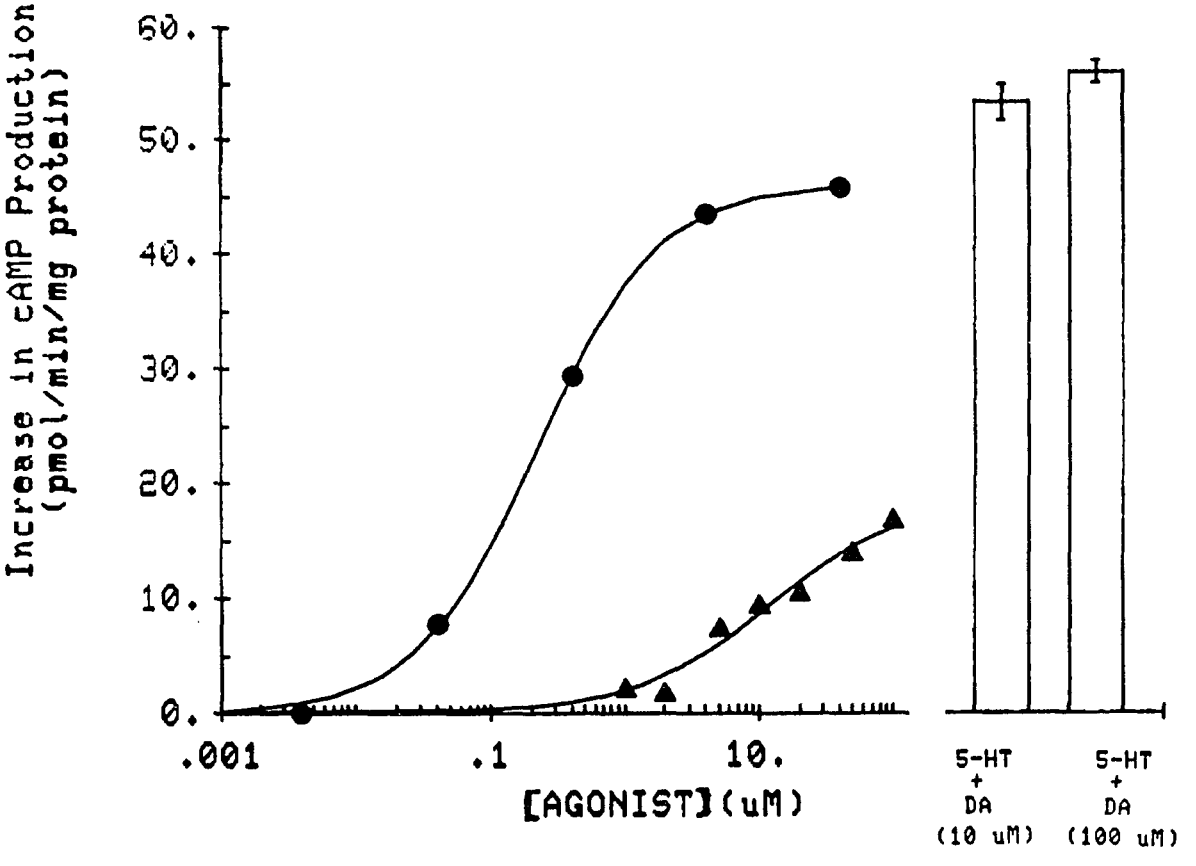

Increase in cAMP Production
(pmol/min/mg protein)
100.
90.
80.
70.
60.
50.
40.
30.
20.
10.
0.
.001
.01
.1
1.
10.
100.
[Agonist] (uM)
5-HT
+
(-)ISO

Fig. 4 Dopamine-stimulated adenylate cyclase activity

Stimulation of adenylate cyclase activity by 5-HT (●) and by dopamine (▲). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 40.0 ± 0.5 pmol cAMP/min/mg protein. The curves are best fits to the logistic function: for 5-HT, EC50 = 218 nM, maximal response = 45.8 pmol cAMP/min/mg protein, slope index = 0.96; for dopamine, EC50 = 12 uM, maximal response = 18.8 pmol cAMP/min/mg protein, slope index = 0.85. Each bar represents the mean increase in cAMP production (± SE) due to a combination of 5-HT and dopamine. The responses elicited by 4 uM 5-HT and 10 uM dopamine are simply additive, but the stimulation produced by a combination of 4 uM 5-HT and 100 uM dopamine is less than expected. Similar results were obtained in several other experiments.


Increase in cAMP Production
(pmol/min/mg protein)
60.
50.
40.
30.
20.
10.
0.
.001
.1
10.
[AGONIST](uM)
5-HT
+
DA
(10 uM)
5-HT
+
DA
(100 uM)

greater than 10 uM is apparently due to activation of 5-HT receptors.

The receptors that mediate stimulation of adenylate cyclase by 5-HT in this preparation appear to be distinct from those that mediate stimulation by other monoamines.

D. Effect of Reserpine

In hippocampal preparations from animals injected with reserpine 25 - 27 hours earlier, 5-HT always produced greater maximal stimulation of adenylate cyclase activity than in preparations from paired animals that had not received reserpine (Fig. 5, upper panel; Table 5). Maximal stimulation by 5-HT averaged 150% over basal activity in membranes from reserpinized animals (range = 106 - 180%). Analysis of the differences between paired parameters (n = 4) revealed that reserpine pretreatment did not significantly affect basal activity, the EC50 value or the slope index of the concentration-response curve of 5-HT (Table 5). In contrast to the increased responsiveness to 5-HT, the response to histamine in the same preparations was unaffected by reserpinization (Fig. 5, lower panel; Table 5).

E. Desensitization in vitro

Preliminary experiments indicated that basal adenylate cyclase activity is linear with respect to time for only 3 - 4 minutes. When membranes and assay components were preincubated together for 8 min at 30° C before the assay

Fig. 5 Effect of reserpine pretreatment on 5-HT- and histamine-stimulated adenylate cyclase activity

Stimulation of adenylate cyclase activity by 5-HT and histamine in hippocampal membranes from a guinea pig injected with reserpine (●) (5 mg/kg, i.p., 25 hours earlier) and from a paired, vehicle-injected control guinea pig (o). Each point represents the mean increase in cAMP production by 5-HT or histamine; the standard error of these values ranged from 0.5 to 1.4 pmol cAMP/min/mg protein. Basal enzyme activity (pmol cAMP/min/mg protein) was 25.8 $\pm$ 0.6 in membranes from the reserpinized animal and 24.2 $\pm$ 0.3 in those from the control. Concentration-response curves for 5-HT had the same EC50 (0.1 uM) and slope index (0.6) for the reserpinized and control animal. The curves for histamine from the two animals had the same EC50 (7 uM) and similar slope indices (reserpinized = 1.0; control = 0.9). This experiment was repeated 3 additional times and results are summarized in Table 5.

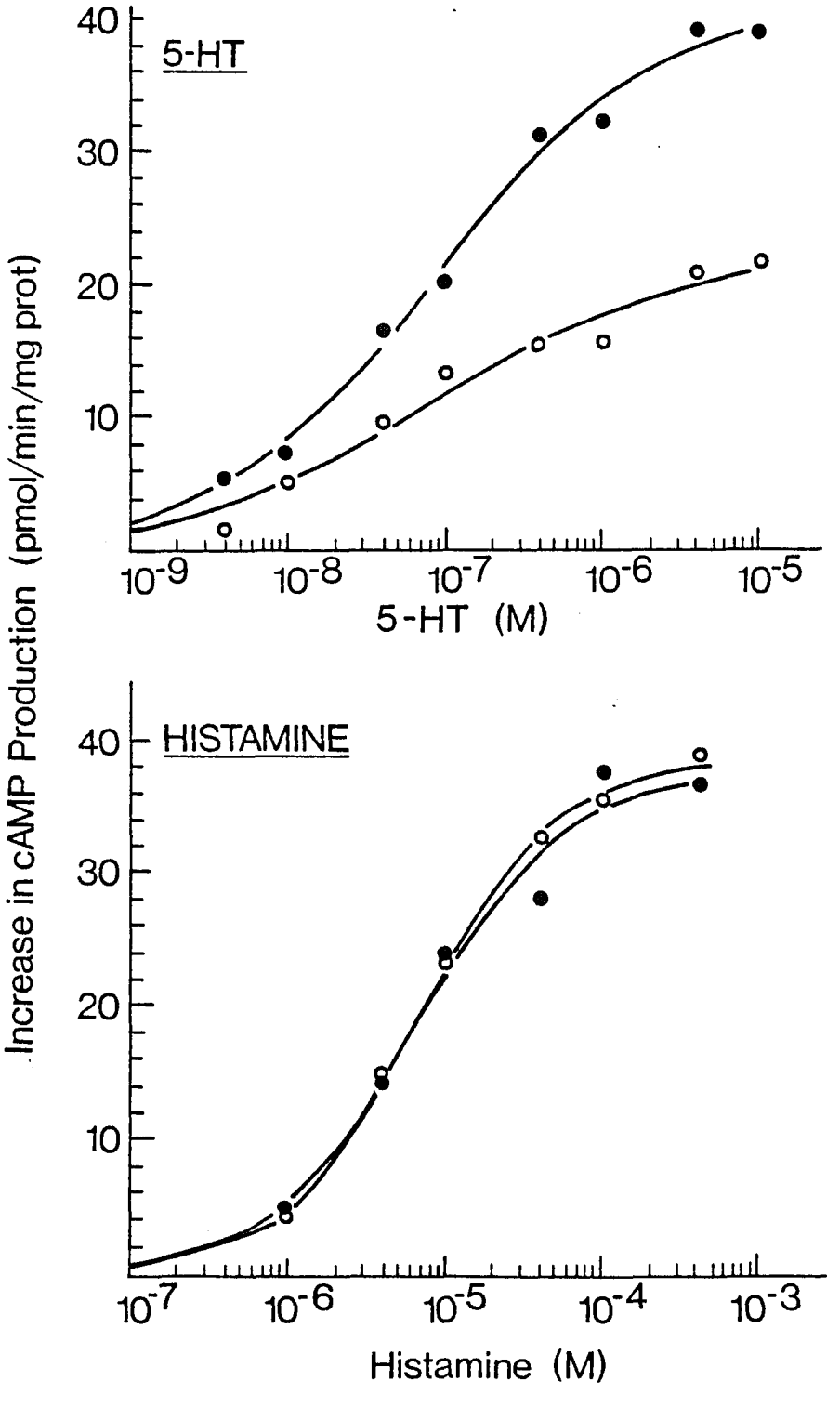

5-HT
40
30
20
10
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
5-HT (M)
HISTAMINE
40
30
20
10
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Histamine (M)
Increase in cAMP Production (pmol/min/mg prot)

Table 5. Effect of reserpine treatment on basal and agonist-stimulated adenylate cyclase activity

PARAMETERS	CONTROL	RESERPINE[a]	DIFFERENCE BETWEEN PAIRED PARAMETERS (reserpine - control)
BASAL ACTIVITY[b]	36.5 ± 6.3	36.3 ± 3.0	- 0.2 ± 0.8
5-HT:			
E_{MAX}[b]	34.2 ± 8.9	55.3 ± 12.0	21.1 ± 3.4[c]
Max % Stimulation	91 ± 11	150 ± 16	59 ± 8[c]
EC_{50}, uM	0.10 ± 0.01	0.13 ± 0.02	0.03 ± 0.23
N (slope index)	0.68 ± 0.07	0.66 ± 0.07	-0.02 ± 0.02
HISTAMINE:			
E_{MAX}[b]	55.6 ± 13.6	60.7 ± 14.3	5.1 ± 2.4
Max % Stimulation	149 ± 15	162 ± 12	13 ± 10
EC_{50}, uM	7.8 ± 1.5	8.3 ± 0.5	0.5 ± 1.1
N (slope index)	0.97 ± 0.04	0.90 ± 0.06	-0.07 ± 0.03

[a] 5 mg/kg, i.p., 25 - 27 hrs before decapitation

[b] pmoles cAMP/min/mg protein

[c] difference is significant by Student's t-test for paired data, $p<0.01$

Each value in the table is mean ± SEM from four separate pairs of control and reserpinized animals. The paired experimental design and the derivation of parameters from concentration-response curves are described in Methods.

(n=11), basal enzyme activity (32.7 ± 2.3 pmol cAMP/min/mg protein) was significantly reduced. 5-HT-stimulated activity declined more rapidly than basal and histamine-stimulated activities. Preincubation with a maximally effective concentration of 5-HT resulted in significantly lower percent stimulation by 5-HT (63 ± 5% over basal activity) than that normally observed. In contrast, maximal percent stimulation by histamine following preincubation with histamine (163 ± 9% over basal activity) was not significantly different from control values.

The 5-HT-dependence of the reduction in responsiveness to 5-HT is illustrated in Table 6. Membranes and assay components were preincubated with or without 5-HT for 30 minutes before starting the assay. The percent stimulation elicited by 10 uM 5-HT in membranes preincubated without 5-HT (90% over basal activity) was about the same as that found in non-preincubated membranes. Preincubation with 5-HT (1 nM - 10 uM) resulted in a concentration-dependent reduction in responsiveness to 10 uM 5-HT. The percent stimulation by 10 uM 5-HT in membranes that had been preincubated with 10 uM 5-HT was only 52% over basal activity. Preincubation with 400 uM histamine for 30 min had no effect on histamine-stimulated adenylate cyclase activity.

The data in Table 6 suggest that the loss of responsiveness to 5-HT is related to 5-HT receptor occupancy, and that it is not simply due to degradation of

Table 6. Effect of preincubation of membranes with agonist on agonist-stimulated adenylate cyclase activity

	[Agonist] (uM)			
	During 30 min Preincubation	Assay	cAMP Production (pmol/min/mg)	Percent Stimulation
BASAL	0	0	25.2 ± 0.5	------
5-HT	0	10	47.5 ± 1.1	88%
	0.001	10	47.8 ± 0.7	90%
	0.01	10	46.8 ± 0.5	86%
	0.1	10	43.3 ± 0.1	72%
	1	10	38.0 ± 0.3	51%
	10	10	38.4 ± 0.4	52%
HISTAMINE	0	400	68.0 ± 1.2	170%
	400	0	64.9 ± 0.5	158%

Data is from one experiment. Tubes containing hippocampal membranes and assay mixture ± agonist were preincubated at 30° C for 30 min; the assay was initiated with [^{32}P]ATP (± concentrations of 5-HT or histamine that elicit maximal stimulation) and conducted for 2 min at 30°. The stimulation elicited by 5-HT in membranes that had been preincubated for 30 min without agonist (88%) was only slightly less than that elicited by 5-HT in membranes that were not preincubated (102%). The reduction in percent stimulation by 5-HT was shown to be dependent on 5-HT (10 uM) in one additional experiment.

5-HT or to denaturation of 5-HT receptors during the preincubation period.

III. Pharmacological Characterization

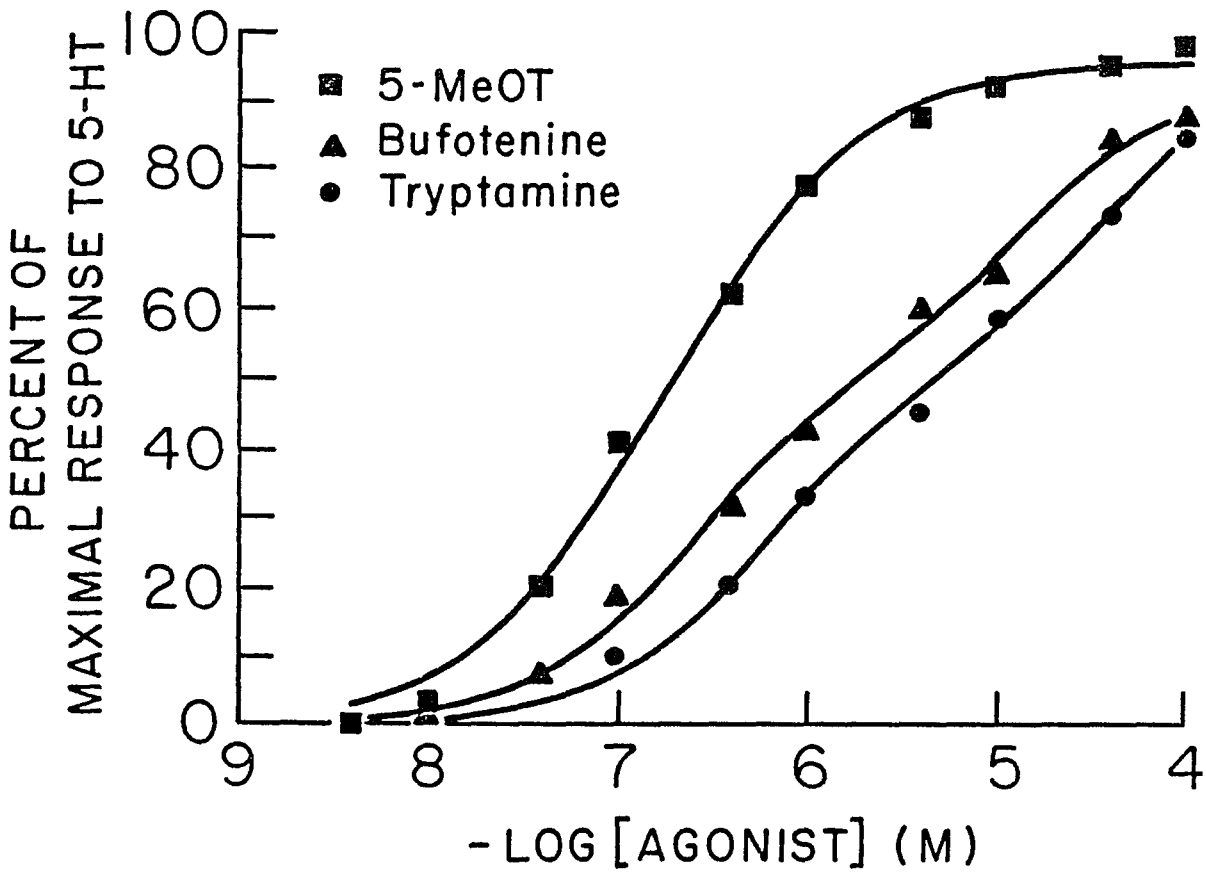
A. Agonists

The 5-HT analogs 5-methoxytryptamine (5-MeOT), 5-hydroxy-N,N-dimethyl-tryptamine (bufotenine), and tryptamine stimulated adenylate cyclase activity (Fig. 6) with mean EC50 values of 193 $\pm$ 39 nM, 620 $\pm$ 158 nM, and 7.1 $\pm$ 1.8 uM, respectively (n=4-6). As shown in Fig. 2, maximal stimulation produced by 5-MeOT or tryptamine was not additive to that produced by 5-HT. The fitted maximal response to bufotenine averaged only 88 $\pm$ 2% of the maximal response to 5-HT; maximal stimulation produced by bufotenine (40 uM) was not additive to that produced by 5-HT (10 uM) (n= 2).

The slope index of the 5-MeOT curve (0.79 $\pm$ 0.04) was indistinguishable from that of 5-HT, unlike the slope indices of the curves for bufotenine (0.65 $\pm$ 0.05) and tryptamine (0.56 $\pm$ 0.02), which were significantly lower. These shallow, non-parallel agonist concentration-response curves raised the possibility that the increase in adenylate cyclase activtiy in this system was mediated by a heterogeneous population of 5-HT receptors, which were better discriminated by bufotenine and tryptamine than by 5-MeOT or 5-HT itself.

Fig. 6 Stimulation of adenylate cyclase activity by 5-MeOT, bufotenine, and tryptamine

Stimulation of adenylate cyclase activity by 5-MeOT (■), bufotenine (▲), and tryptamine (●). The data were compiled from two different experiments and are expressed as a percentage of the maximal stimulation by 5-HT in each experiment. The curves are the best fits to equation (1); $Emax_H$ was constrained to 50% on the basis of mean 5-$CONH_2$-T data. The fits provided the following parameter estimates: for 5-MeOT: K_H = 72 nM, K_L = 516 nM, $Emax_L$ = 46%; for bufotenine: K_H = 233 nM, K_L = 14.8 uM, $Emax_L$ = 44%; for tryptamine: K_H = 584 nM, K_L = 42.0 uM, $Emax_L$ = 49%.

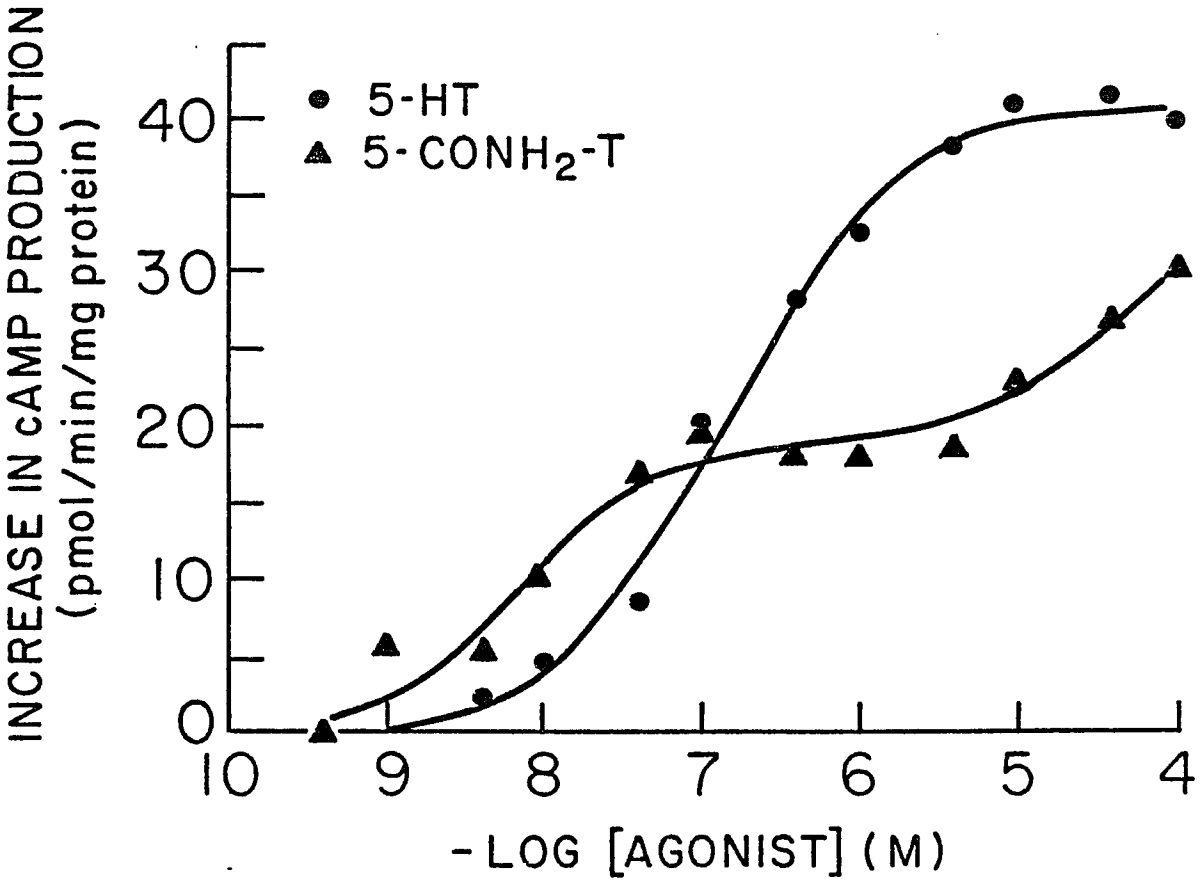

PERCENT OF
MAXIMAL RESPONSE TO 5-HT
100
80
60
40
20
0
9
8
7
6
5
4
5-MeOT
Bufotenine
Tryptamine
-LOG [AGONIST] (M)

5-Carboxamidotryptamine (5-$CONH_2$-T) produced a distinctly biphasic concentration-response curve (Fig 7). The first component of the curve lay to the left of the 5-HT curve and reached a plateau at 5-$CONH_2$-T concentrations of 0.1 - 4 uM. The plateau represented 49 $\pm$ 2% (n = 30) of the maximal response to 5-HT. Higher concentrations of 5-$CONH_2$-T elicited a further increase in the rate of cAMP production. Fitting the initial component of 5-$CONH_2$-T curves (4 nM - 4 uM; n = 13) to the logistic function yielded a mean EC50 of 6 $\pm$ 1 nM and a slope index (1.04 $\pm$ 0.04) not significantly different from 1.0, suggesting activation of only a single receptor type. Although the second component of the curve did not clearly reach a plateau, it is evident that 5-$CONH_2$-T is at least 5000 times less potent in eliciting this part of the response. Stimulation produced by 100 uM 5-$CONH_2$-T was not additive to the maximal stimulation produced by 5-HT (n = 4; legend to Fig. 7), indicating that the entire response to 5-$CONH_2$-T was mediated by the same receptors that mediated the response to 5-HT.

These data led to the hypothesis that guinea pig hippocampal membranes contain two distinct 5-HT receptors coupled to adenylate cyclase, each contributing about 50% to the total response. The predictions of this two-receptor model were then tested.

Fig. 7 Stimulation of adenylate cyclase activity by 5-HT and 5-$CONH_2$-T

Stimulation of adenylate cyclase activity by 5-HT (●) and 5-$CONH_2$-T (▲). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 42.5 $\pm$ 0.9 pmol cAMP/min/mg protein. The stimulation produced by a combination of 4 uM 5-HT and 100 uM 5-$CONH_2$-T (38.7 $\pm$ 0.8 pmol cAMP/min/mg protein) was not significantly different from that produced by 4 uM 5-HT alone. The curves are the best fits to equation (1); the data for 5-HT and 5-$CONH_2$-T were fit simultaneously with a common value for $Emax_H$. The fit provided the following parameter estimates: $Emax_H$ = 18.6 pmol cAMP/min/mg protein; for 5-HT: K_H = 42 nM, K_L = 412 nM, $Emax_L$ = 21.8 pmol cAMP/min/mg protein; for 5-$CONH_2$-T: K_H = 7 nM, K_L = 42.0 uM, $Emax_L$ = 15.9 pmol cAMP/min/mg protein. In this experiment, 46% of the maximal response was due to activation of R_H.

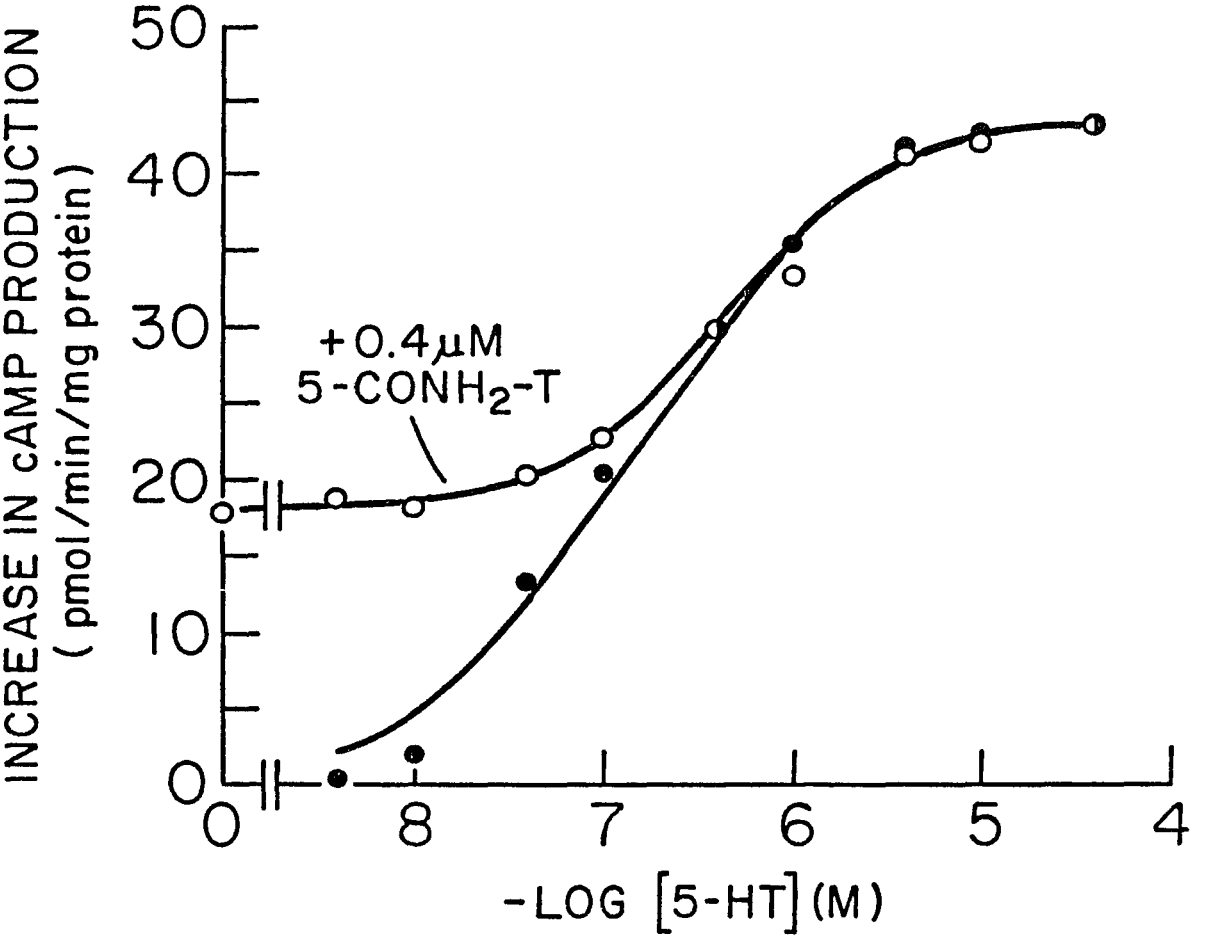

INCREASE IN cAMP PRODUCTION
(pmol/min/mg protein)
40
30
20
10
0
5-HT
5-CONH2-T
10
9
8
7
6
5
4
-LOG [AGONIST] (M)

B. The Two Receptor Model

If 0.4 uM 5-$CONH_2$-T elicits maximal stimulation through one receptor population without significantly affecting the second (see Fig. 7), then stimulation of adenylate cyclase activity by another agonist in the presence of 0.4 uM 5-$CONH_2$-T should appear monophasic and reflect activation only of the second receptor population. The results of a representative experiment of this design are shown in Fig.8. The concentration-response curve for 5-HT in the presence of 0.4 uM 5-$CONH_2$-T is nearly superimposible on the upper portion of the 5-HT curve. As predicted, the mean slope index of such curves (0.88 $\pm$ 0.08, n=4) was not significantly different from 1.0. From these results it was deduced that the initial, high affinity components of the curves for 5-$CONH_2$-T and 5-HT are mediated by the same receptor. For purposes of discussion, this receptor is referred to as R_H. The receptor mediating the second, low affinity components of the curves is referred to as R_L. Experiments analogous to the one shown in Fig 8 performed with 5-MeOT, tryptamine and bufotenine (e.g., Fig. 12) revealed that these three agonists were also selective for R_H. Consequently, concentration-response data were fit to a model describing the action of an agonist at two independent receptors that mediate the same response (Ariens et al., 1956; Hough et al., 1980):

Fig. 8 Stimulation of adenylate cyclase activity by 5-HT in the presence of 0.4 uM 5-$CONH_2$-T

Stimulation of adenylate cyclase activity by 5-HT alone (●) and by 5-HT in the presence of 0.4 uM 5-$CONH_2$-T (○). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 37.2 ± 0.5 pmol cAMP/min/mg protein. The curves are the result of simultaneously fitting the response data for 5-HT alone and for 5-HT plus 5-$CONH_2$-T to equation (1) and equation (2), respectively. It was assumed that 5-$CONH_2$-T was a full agonist on R_H ($\beta_H = 1.0$). The apparent affinities of 5-$CONH_2$-T for R_H and R_L were fixed, based on average results; the effect of 0.4 uM 5-$CONH_2$-T on R_L is negligible. The fit provided the following parameter estimates: for 5-HT, K_H = 32 nM, K_L = 447 nM; $Emax_H$ = 18.4 pmol cAMP/min/mg protein; $Emax_L$ = 24.9 pmol cAMP/min/mg protein. Each curve was also fit individually to the logistic function (curves not shown). The curve for 5-HT alone had an EC50 of 137 nM and a slope index of 0.82. The curve for 5-HT in the presence of 5-$CONH_2$-T had an EC50 of 554 nM and a slope index of 0.92. Similar results were obtained in three additional experiments. Preincubating the agonists and membranes together for 8 min did not affect the shape or relative position of the curves.


INCREASE IN cAMP PRODUCTION
(pmol/min/mg protein)
50
40
30
20
10
0
+0.4μM
5-CONH2-T
0
8
7
6
5
4
-LOG [5-HT](M)

$$E = Emax_H[A]/(K_H + [A]) + Emax_L[A]/(K_L + [A]) \quad (1)$$

where E represents agonist-stimulated cyclase activity; $Emax_H$ and $Emax_L$ represent the maximal stimulation due to R_H and R_L, respectively; [A] represents agonist concentration; and K_H and K_L represent the EC50 values of the agonist at R_H and R_L, respectively. It was assumed that responses due to the activation of R_H and R_L were strictly additive, as additivity of responses mediated by different receptors has been demonstrated in this system (Figs. 2, 3, 4). Once the apparent selectivity of 5-$CONH_2$-T was established, $Emax_H$ was estimated by measuring the stimulation produced by 0.4 uM 5-$CONH_2$-T in each experiment. Data from earlier experiments were generally analyzed by constraining the percentage of maximal response to 5-HT mediated by R_H to be 50%, based on the average results with 5-$CONH_2$-T. Estimates of K_H and K_L generated by a computerized, nonlinear least squares curve fitting procedure (Baig and Reid-Miller, 1980) are summarized in Table 7. Fitted curves are illustated in Figs 6 and 7. As expected from the two-receptor model, the mean K_L of each agonist estimated by computer fit was comparable to the concentration of that agonist that produced half-maximal stimulation in the presence of 0.4 uM 5-$CONH_2$-T.

Based on the fitted estimates of $Emax_L$ for 5-HT and 5-$CONH_2$-T, the calculated intrinsic activity of 5-$CONH_2$-T at

Table 7. Potencies of agonists at two 5-HT receptors coupled to adenylate cyclase[a]

Agonist	n	K_H (nM)	K_L (nM)
5-HT	15	43 ± 6	414 ± 53
5-MeOT	4	51 ± 12	578 ± 107
Bufotenine	6	123 ± 25	4,790 ± 1,640[b]
Tryptamine	5	592 ± 142	33,100 ± 9,600
5-$CONH_2$-T	13	6 ± 1	31,300 ± 4,500[b]
8-OH-DPAT	4	29 ± 11[c]	>50,000
Buspirone	3	200 ± 92[c]	>50,000

[a] Each value is the geometric mean ± SE of parameters estimated by computer fit, as explained in the text.

[b] May be a partial agonist at R_L; the K_L of 5-$CONH_2$-T is based on data from the six experiments where concentrations of 5-$CONH_2$-T as high as 100 uM were used.

[c] Partial agonist at R_H.

R_L is 0.8 $\pm$ 0.1. Because the second component of the 5-$CONH_2$-T curve did not reach a plateau within the concentration range studied, however, it is unclear whether 5-$CONH_2$-T is actually a partial agonist at R_L.

C. Competitive Interaction of Two Drugs at Two Receptors

To further investigate the validity of the two receptor model, data from experiments in which the response to an agonist, A, was measured in the presence of a single concentration of another drug, B, were fit to a general model describing the competitive interaction of two drugs (Van den Brink, 1977) at two independent receptors mediating the same response:

$$E = \frac{\alpha_H Emax_H[A]}{K_H(1 + [B]/KB_H) + [A]} + \frac{\beta_H Emax_H[B]}{KB_H(1 + [A]/K_H) + [B]} + \frac{\alpha_L Emax_L[A]}{K_L(1 + [B]/KB_L) + [A]} + \frac{\beta_L Emax_L[B]}{KB_L(1 + [A]/K_L) + [B]} \quad (2)$$

where E represents agonist-stimulated cyclase activity; $Emax_H$ and $Emax_L$ represent the maximal stimulation due to R_H and R_L, respectively; K_H and K_L represent the dissociation constants of A for R_H and R_L, respectively; α_H and α_L represent the intrinsic activities of A at R_H and R_L, respectively; KB_H and KB_L represent the dissociation constants of B for R_H and R_L, respectively; β_H and β_L

represent the intrinsic activities of B at R_H and R_L, respectively.

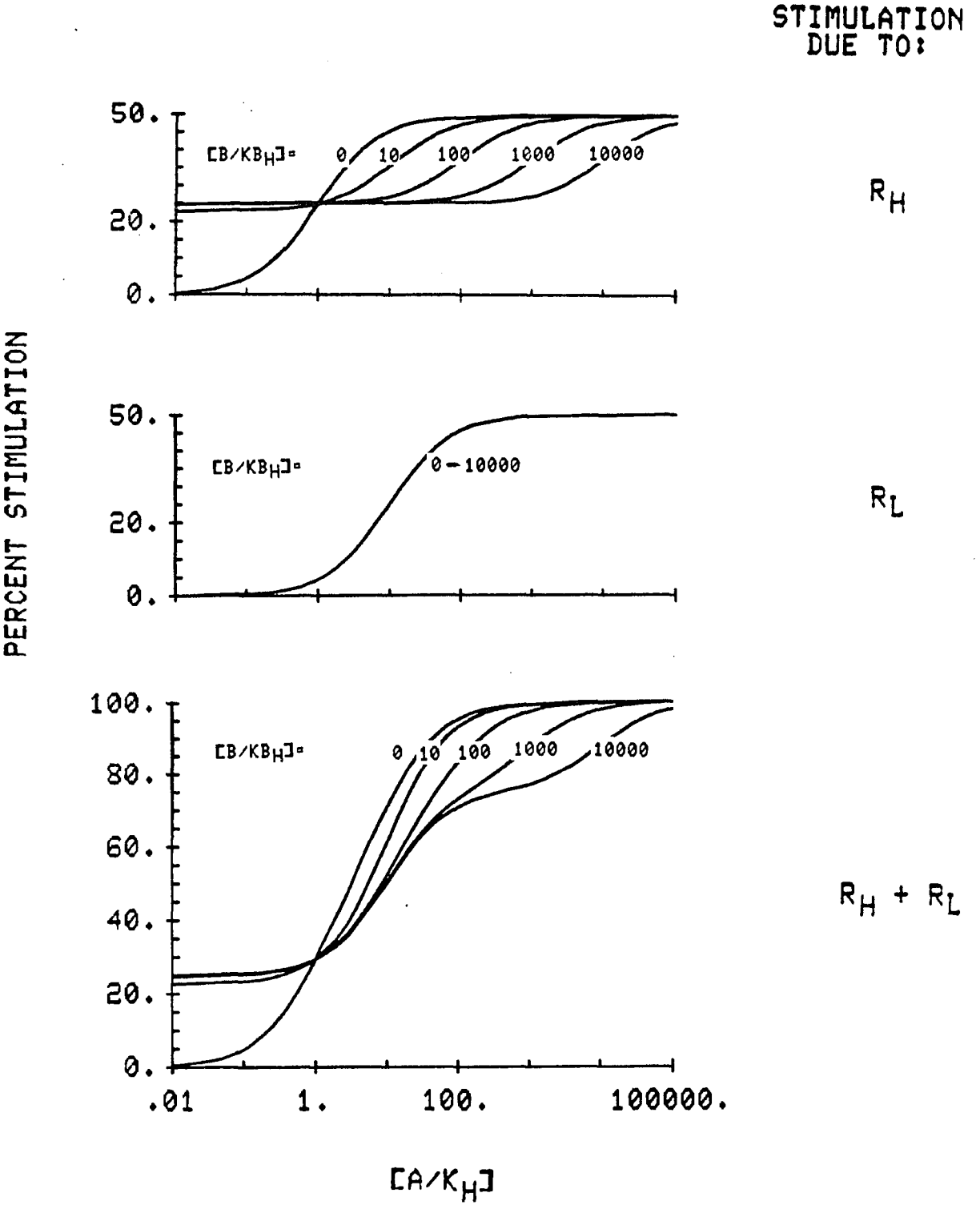
To use this model of interaction, the simplifying assumption was made that the dissociation constant of an agonist was equivalent to its EC50 value, i.e., that there were no "spare receptors" in this system.

1) The intrinsic activity of 5-$CONH_2$-T at R_H

Data on the response to 5-HT in the presence of 0.4 uM 5-$CONH_2$-T were adequately fit by equation (2) when 5-$CONH_2$-T was assumed to be a full agonist at R_H (Fig. 8). Simulations support this assumption; a selective partial agonist at R_H should cause an apparently complex rightward shift of the upper part of the 5-HT curve, a result that was not observed. A representative simulation is shown in Fig. 9. The shift of the concentration-response curve to drug A that is produced by drug B is understood by separately considering the effect that B has on the component of response mediated by R_H, and the lack of effect it has on the component of response mediated by R_L. In the presence of increasing concentrations of B, the component of response mediated by R_L does not move. In the presence of 10,000 KB_H of B, (Fig. 9), the first component of the response (25% stimulation) is due to activation of R_H; the "middle" component (50% stimulation) is due to activation of R_L; the final component (25% stimulation) is due to activation of R_H. It is interesting to note that if drug B were also to act as an antagonist at R_L, the effect would be seen as a

Fig. 9 Simulated effect of a partial agonist at R_H on the concentration-response curve to a drug that is a full agonist at R_H and R_L

Simulated interaction of an agonist, A, possessing 10-fold selectivity for R_H ($K_H = 1$, $K_L = 10$) with a drug, B, that is a partial agonist at R_H ($KB_H = 1$, $\beta_H = 0.5$) and that has no affinity for R_L ($KB_L = \infty$). Maximal stimulation mediated by each receptor is 50% of the total response. The figure shows the response to A alone and A in the presence of increasing concentrations of B ($[B/KB_H] = 0$, 10, 100, 1000, 10,000).


STIMULATION DUE TO:
PERCENT STIMULATION
50.
20.
0.
[B/KB$_H$]=
0
10
100
1000
10000
R_H
50.
20.
0.
[B/KB$_H$]=
0-10000
R_L
100.
80.
60.
40.
20.
0.
[B/KB$_H$]=
0
10
100
1000
10000
$R_H + R_L$
.01
1.
100.
100000.
[A/K$_H$]

shift of the "middle" component to the right. This point is addressed in experiments with 8-OH-DPAT which will be described later.

2) Response to 5-$CONH_2$-T in the presence of 5-HT

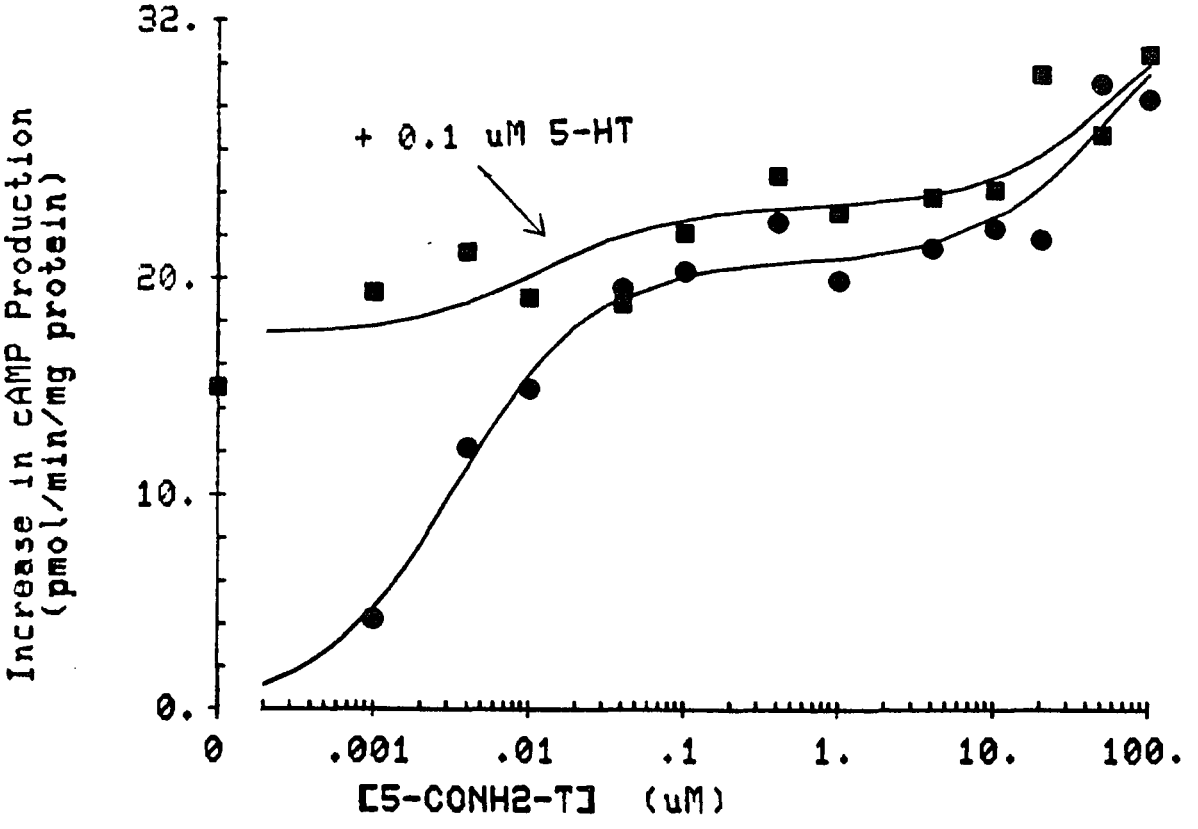
The response to 5-$CONH_2$-T measured in the presence of 0.1 uM 5-HT was complex (Fig. 10); the fact that these data were satisfactorily fit by the two receptor model provides further evidence for its validity. Based on the fitted estimates of K_H and K_L for 5-HT (Table 7), the stimulation elicited by 0.1 uM 5-HT represents 70% of $Emax_H$ plus 20% of $Emax_L$. Low concentrations of 5-$CONH_2$-T act synergistically with 0.1 uM 5-HT in eliciting stimulation of R_H. The observed "plateau" of stimulation corresponds to maximal stimulation through R_H plus 20% of the maximal stimulation through R_L. Concentrations of 5-$CONH_2$-T greater than 10 uM elicit additional stimulation by activating R_L.

3) Intrinsic activity of bufotenine

When the data for bufotenine were first analyzed, it was assumed that the low maximal response relative to that of 5-HT was due to partial agonism at R_L (see legend to Fig. 6). The alternative possibility that bufotenine was a partial agonist at R_H was evaluated. Simulations based on equation (2) indicated that the two models were theoretically distinguishable by measuring response to bufotenine (drug A) alone and in the presence of a concentration of 5-$CONH_2$-T (drug B) that elicits maximal

Fig. 10 Stimulation of adenylate cyclase activity by 5-$CONH_2$-T in the presence of 0.1 uM 5-HT

Stimulation of adenylate cyclase activity by 5-$CONH_2$-T alone (●) and by 5-$CONH_2$-T in the presence of 0.1 uM 5-HT (■). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 48.8 ± 1.0 pmol cAMP/min/mg protein. The curves are the result of simultaneously fitting the response data for 5-$CONH_2$-T alone and for 5-$CONH_2$-T plus 5-HT to functions similar to equation (1) and equation (2), respectively. On the basis of data with 5-HT in this experiment, maximal response due to R_H plus R_L was set to 34.7 pmol cAMP/min/mg protein. The fit provided the following parameter estimates: proportion of maximal response due to R_H = 60%; for 5-$CONH_2$-T: K_H = 4 nM, K_L = 61 uM; for 5-HT: KB_H = 40 nM, KB_L = 449 nM.


32.
20.
10.
0.
Increase in cAMP Production
(pmol/min/mg protein)
+ 0.1 uM 5-HT
0
.001
.01
.1
1.
10.
100.
[5-CONH2-T] (uM)

stimulation through R_H (Fig. 11). In practice, the differences were difficult to detect because of scatter in the data and because of the relatively high intrinsic activity of bufotenine. The experiment shown in Fig. 12 produced the least ambiguous results. Comparison of the fits shown in Panel I and II suggests that the model in which bufotenine is a partial agonist at R_L provides a more satisfactory explanation of the data than the model in which bufotenine is a partial agonist at R_H. The data from another experiment in which the response to bufotenine was measured in the presence of 4 uM 5-$CONH_2$-T were also better fit by the model in which bufotenine was assumed to be a partial agonist at R_L, although the difference was not as marked as in Fig. 12. In a third experiment, in which the response to bufotenine was measured in the presence of 0.4 uM 5-$CONH_2$-T, the two models appeared to fit the data equally well.

D. Antagonism by Spiperone

As illustrated in Fig. 13, spiperone inhibited 5-HT-stimulated adenylate cyclase activity (n = 5 experiments). Spiperone alone generally caused a 10-20% decrease in basal enzyme activity, but this effect was not clearly concentration-related; the decreased basal values were used in calculating net stimulation by 5-HT. On two occasions 5-HT and spiperone were incubated together with

Fig. 11 Simulated interactions of two agonists at two receptors mediating the same response: partial agonism at R_H or R_L

I. Simulated response to a drug, A, that is a partial agonist at R_H ($\alpha_H = 0.8$) and a full agonist at R_L ($\alpha_L = 1.0$). A is 50-fold selective for R_H ($K_H = 1$, $K_L = 50$). Maximal stimulation mediated by each receptor is 50% of the total response. Drug B is a full agonist at R_H ($KB_H = 1$, $\beta_H = 1.0$) and has no affinity for R_L. The figure shows the response to A alone and to A in the presence of concentrations of B that elicit maximal stimulation through R_H ($[B/KB_H] = 0, 100, 1000$).

II. Same description as I, except here A is a full agonist at R_H ($\alpha_H = 1.0$) and a partial agonist at R_H ($\alpha_L = 0.8$).

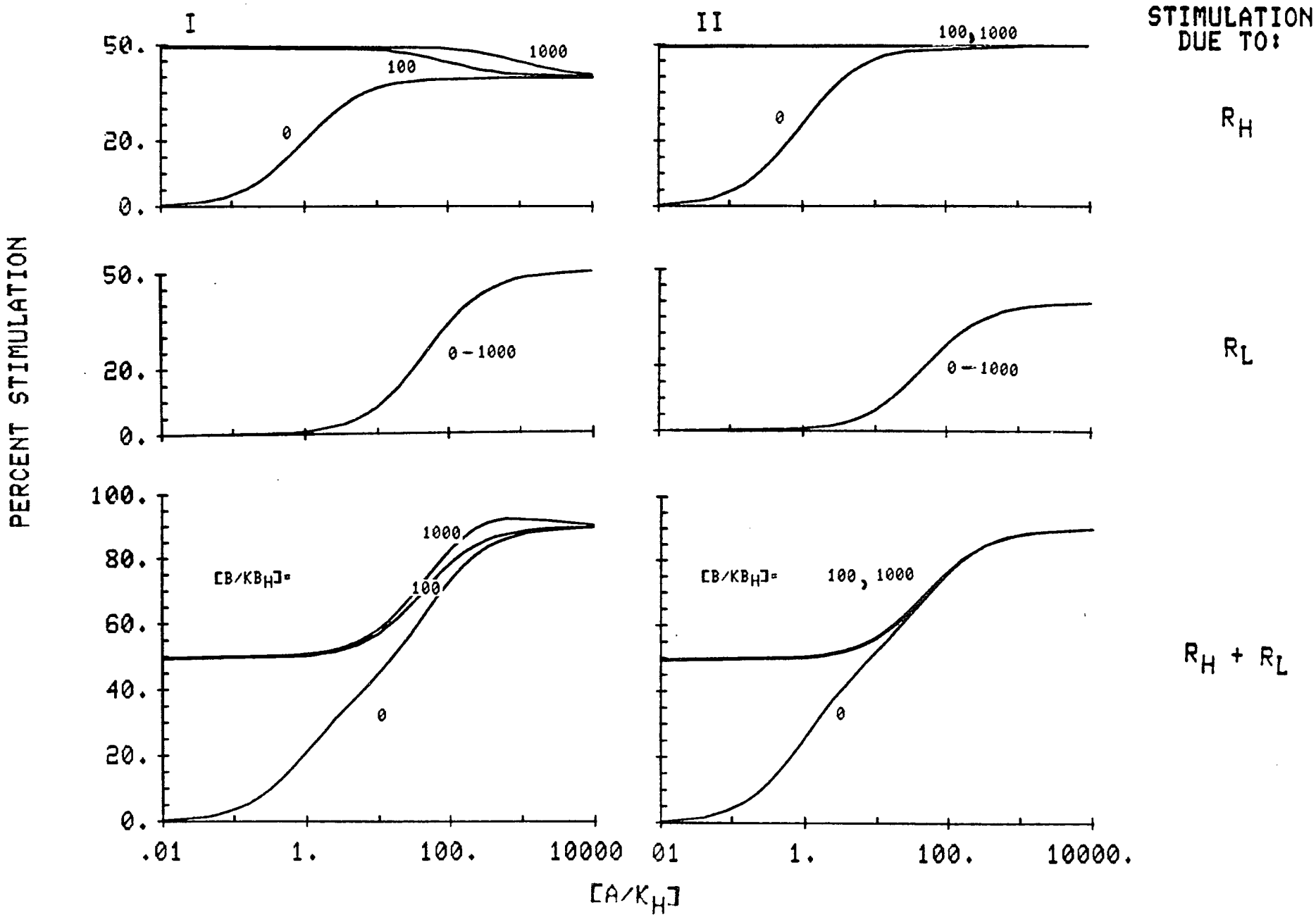

I
II
STIMULATION DUE TO:
R_H
R_L
$R_H + R_L$
PERCENT STIMULATION
50.
20.
0.
100.
80.
60.
40.
1000
100
0
100, 1000
0 - 1000
[B/KB$_H$]=
.01
1.
100.
10000
[A/K$_H$]

Fig. 12 Stimulation of adenylate cyclase activity by bufotenine in the presence of 0.4 uM 5-$CONH_2$-T: comparison of the fits to two different models

Stimulation of adenylate cyclase activity by bufotenine alone (●) and by bufotenine in the presence of 0.4 uM 5-$CONH_2$-T (▲). The data were fit to equations based on a model in which bufotenine was assumed to be a partial agonist at R_H and a full agonist at R_L (Panel I), and to equations based on a model in which bufotenine was assumed to be a full agonist at R_H and a partial agonist at R_L (Panel II). In each case, the data on stimulation by bufotenine alone ([B] = 0) and by bufotenine in the presence of 5-$CONH_2$-T ([B] = 0.4 uM) were fit simultaneously to two functions in the form of equation (2). Based on results with 5-HT and 5-$CONH_2$-T in this experiment, $Emax_H$ = $Emax_L$ = 22 pmol cAMP/min/mg protein. Based on average data with 5-$CONH_2$-T, KB_H was set to 7 nM. The effect of 0.4 uM 5-$CONH_2$-T on R_L is negligible.

I. In this case, α_L was set to 1.0. The fit provided the following parameter estimates for bufotenine: K_H = 63 nM, K_L = 3.5 uM, α_H = 0.72. Residual sum of squares was 70.

II. In this case, α_H was set to 1.0. The fit provided the following parameter estimates for bufotenine: K_H = 126 nM, K_L = 4.5 uM, α_L = 0.69. Residual sum of squares was 44.

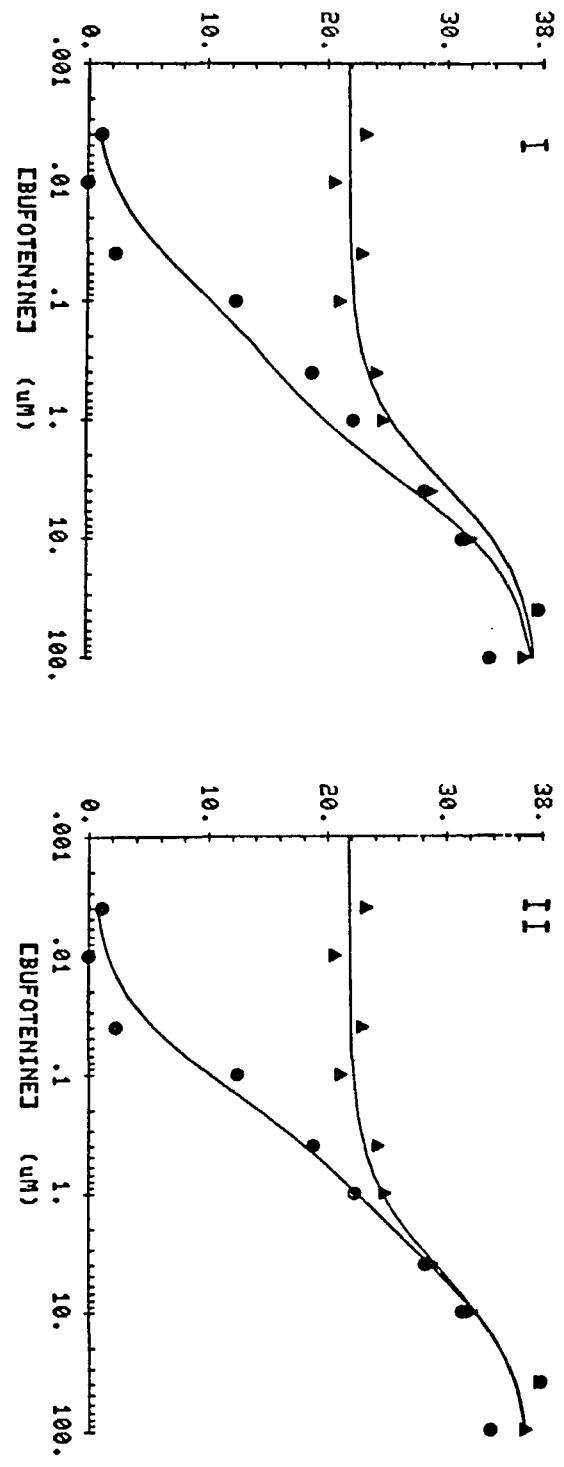
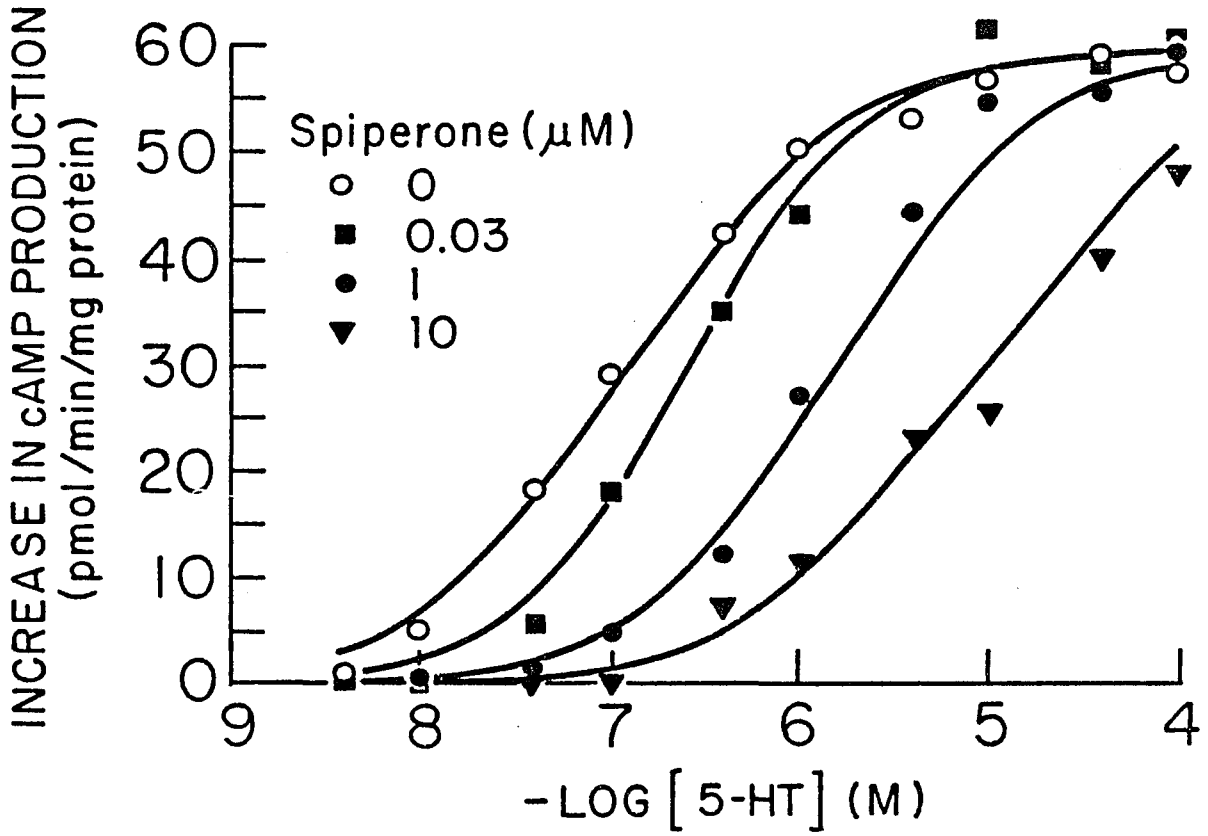

Increase in cAMP Production
(pmol/min/mg protein)
0.
10.
20.
30.
38.
I
II
.001
.01
.1
1.
10.
100.
[BUFOTENINE] (uM)

Fig. 13 Antagonism of 5-HT by spiperone

Stimulation of adenylate cyclase activity by 5-HT alone (○) and by 5-HT in the presence of 30 nM (■), 1 uM (●), and 10 uM (▼) spiperone. Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 56.4 ± 1.5 pmol cAMP/min/mg protein. Spiperone alone caused small decreases (≤ 10%) in basal activity; these decreased values were used in calculating net stimulation by 5-HT. Spiperone had no effect on maximal stimulation by 5-HT in this experiment. Parallelism of the concentration-response curves was assessed as described in Methods. The estimated slope indices provided by the variable slope fit (curves not shown) were 0.82, 0.99, 1.06, and 0.63 for the curves in the presence of 0, 30 nM, 1 uM and 10 uM spiperone, respectively. The more complex variable slope model fit these data significantly better than the common slope model ($F_{(3,31)} = 11.5$). The data were then fit simultaneously to four functions in the form of equation (3) (curves shown in figure). The mean stimulation by 0.4 uM 5-$CONH_2$-T in this experiment (28.7 pmol cAMP/min/mg protein) was used as an estimate of $Emax_H$. The fit provided the following parameter estimates: $Emax_L$ = 29.7 pmol cAMP/min/mg protein; for 5-HT: K_H = 38 nM, K_L = 392 nM; for spiperone: KB_H = 9 nM, KB_L = 2.1 uM.


INCREASE IN cAMP PRODUCTION
(pmol/min/mg protein)
60
50
40
30
20
10
0
Spiperone (μM)
0
0.03
1
10
9
8
7
6
5
4
-LOG [5-HT] (M)

the hippocampal membranes for 8 min before conducting the assay; in one of these experiments the maximal response to 5-HT was increased 20% in the presence of spiperone. In that experiment the data were expressed as a percent of the maximal response to 5-HT for each curve before further analysis. The results did not appear to vary with time of incubation or data tranformation.

Spiperone (30 nM - 40 uM) shifted the 5-HT concentration-response curve to the right in a manner that was surmountable, concentration-dependent, but markedly non-parallel. In four of five experiments the results of a partial F-test indicated that the data were fit significantly better by the more complex "variable slope" model than by the "common slope" model (e.g., legend to Fig. 13). Concentration- response curves for 5-HT obtained in the presence of $\leq$ 1 uM spiperone were consistently steeper than the control curve, while those obtained in the presence of $\geq$ 10 uM spiperone were shallower. If the non-parallelism of the 5-HT curves in the presence of spiperone is ignored and a Schild plot is constructed from fits to the "common slope" model, the slope of the plot (0.78 $\pm$ 0.07) is significantly less than 1.0. The conditions of the assay (non-physiological buffer, inclusion of pargyline, two min incubation at 30° C) make it very unlikely that the atypical antagonism was due to uptake or metabolism of 5-HT (Kenakin, 1982). Although the variable slope model provided a good fit of the data, it lacks operational meaning.

It was determined that the two-receptor model provides a satisfactory explanation for the atypical antagonism of 5-HT by spiperone. The set of curves from each experiment were fit simultaneously to equations of the form:

$$E = \frac{Emax_H\,[A]}{K_H(1 + [B]/KB_H) + [A]} + \frac{Emax_L\,[A]}{K_L(1 + [B]/KB_L) + [A]} \quad (3)$$

where E represents agonist-stimulated cyclase activity; $Emax_H$ and $Emax_L$ represent the maximal stimulation due to R_H and R_L, respectively; [A] represents agonist concentration; K_H and K_L represent the EC50 values of agonist A for R_H and R_L, respectively; [B] represents antagonist concentration; KB_H and KB_L represent the dissociation constants of the antagonist B at R_H and R_L, respectively. Equation (3) is a simplified form of equation (2) where drug B has zero intrinsic activity at R_H and R_L, i.e., it is an antagonist. For curve-fitting, the value of $Emax_H$ was fixed on the basis of results with 5-$CONH_2$-T.

The results of this analysis indicate that spiperone has considerably higher affinity for R_H (KB_H = 14 $\pm$ 4 nM) than for R_L (KB_L = 2 $\pm$ 1 uM). The higher affinity of spiperone for the receptor mediating the initial component of the 5-HT curve is manifest in the steepening of the curve that occurs in the presence of low concentrations of spiperone. Since the component of the curve mediated by R_L is not initially affected, the relative positions of the two

components are reversed as spiperone concentration is increased (i.e., $K_H(1 + [spiperone]/KB_H > K_L)$. The decreased slope of the 5-HT curve in the presence of high concentrations of spiperone is a result of the upper component of the curve (now mediated by R_H) being shifted proportionately more than the lower component.

A computer-generated simulation of the effects of a selective antagonist on a response due to the activation of two separate receptors is shown in Fig. 14; these effects were previously illustrated by Hough and co-workers (1980). The responses due to activation of R_H and R_L are shown separately, as is the net response that would be observed in a system containing a mixed population of R_H and R_L, each responsible for 50% of the maximal response.

Atypical antagonism of bufotenine by 100 nM spiperone (Fig. 15) was also explained by the two-receptor model. Bufotenine was assumed to be a full agonist at R_H (see earlier results). The fitted estimate of the dissociation constant of spiperone for R_H (30 nM) was comparable to the value obtained when 5-HT was the agonist.

In contrast to its complex antagonism of 5-HT (Fig. 13), spiperone (30 nM - 10 uM) was a simple competitive antagonist of the first component of the 5-$CONH_2$-T curve (Fig. 16). No deviation from parallelism was observed in five separate experiments. The slope of the Schild plot constructed from these experiments (1.10 $\pm$ 0.08) was not significantly different from 1.0. The intercept of a line

Fig. 14 Simulated effect of a selective antagonist on a response due to activation of two separate receptors

Simulated inhibition of response to an agonist, A, with 10-fold selectivity for R_H ($K_H = 1$, $K_L = 10$) by a competitive antagonist, B, with 1000-fold selectivity for R_H ($KB_H = 1$, $KB_L = 1000$). Maximal stimulation mediated by each receptor is 50% of the total response. The figure shows response to A alone and to A in the presence of increasing concentrations of B ($[B/KB_H] = 0, 1, 10, 100, 1000, 10000$).

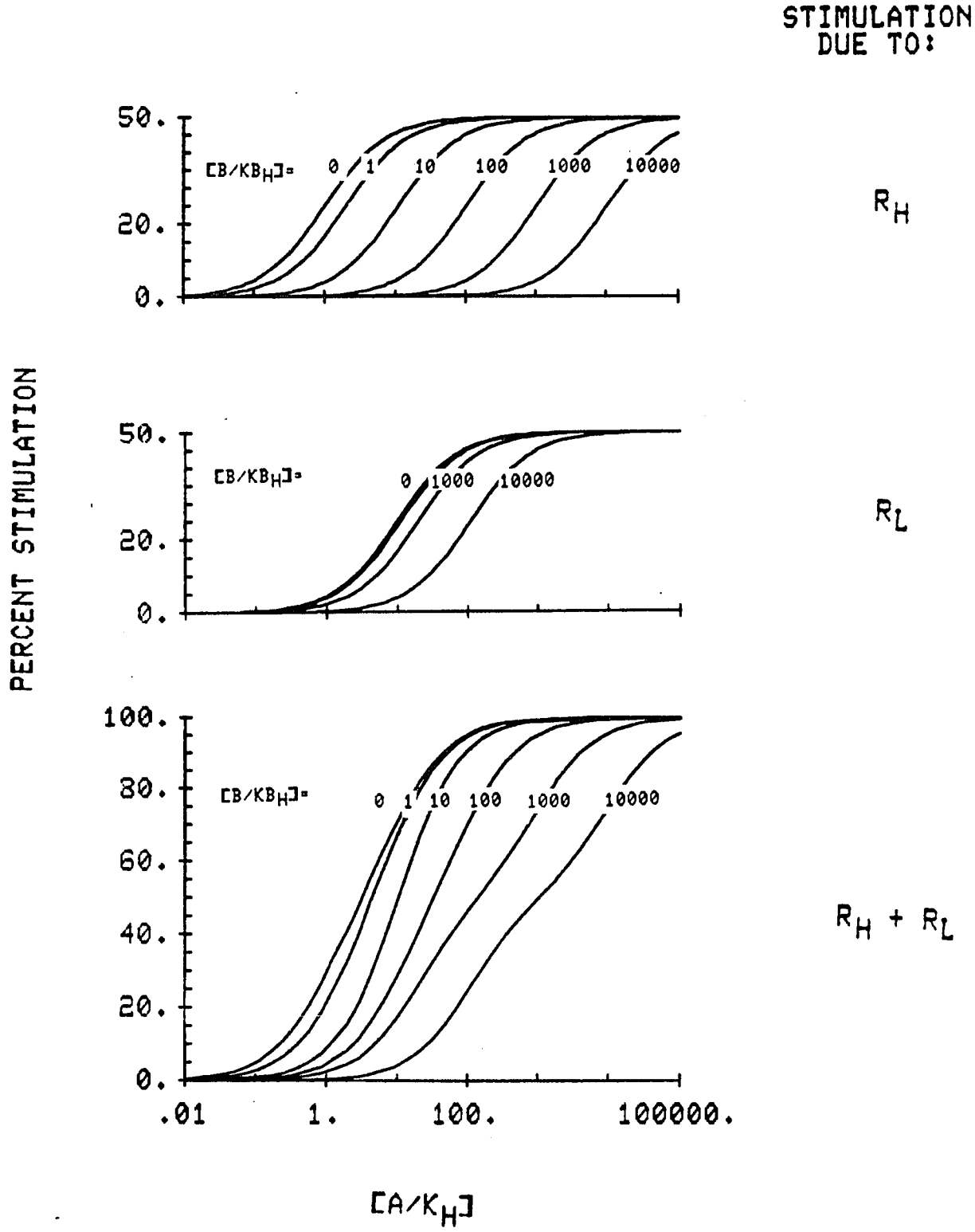

STIMULATION DUE TO:
PERCENT STIMULATION
50.
20.
0.
[B/KB_H]=
0
1
10
100
1000
10000
R_H
50.
20.
0.
[B/KB_H]=
0
1000
10000
R_L
100.
80.
60.
40.
20.
0.
[B/KB_H]=
0
1
10
100
1000
10000
R_H + R_L
.01
1.
100.
100000.
[A/K_H]

Fig. 15 Antagonism of bufotenine by 0.1 uM spiperone

Stimulation of adenylate cyclase activity by bufotenine alone (●) and by bufotenine in the presence of 0.1 uM spiperone (▲). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 43.7 $\pm$ 0.8 pmol cAMP/min/mg protein; spiperone alone caused a 6% decrease in basal activity. The variable slope model, which fit these data significantly better than the common slope model ($F_{(1,15)} = 5.3$), provided the following slope index estimates: bufotenine alone, 0.71; bufotenine plus spiperone, 0.89 (curves not shown). The data were fit simultaneously to two functions in the form of equation (3) (curves shown in figure). The mean stimulation by 0.4 uM 5-$CONH_2$-T in this experiment (20.5 pmol cAMP/min/mg protein) was used an an estimate of $Emax_H$. The fit provided the following parameter estimates: $Emax_L$ = 15.3 pmol cAMP/min/mg protein; for bufotenine: K_H = 160 nM, K_L = 3.4 uM; for spiperone: KB_H = 30 nM, KB_L = "undefined".

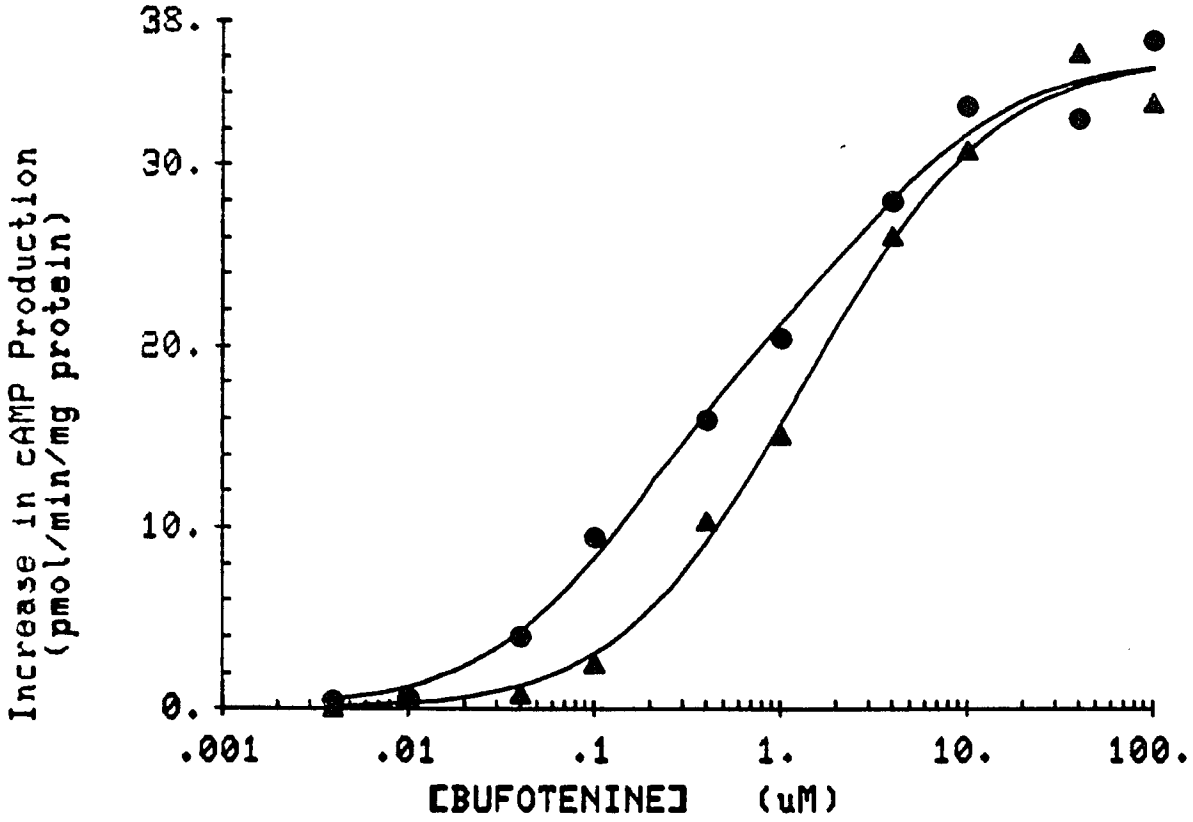
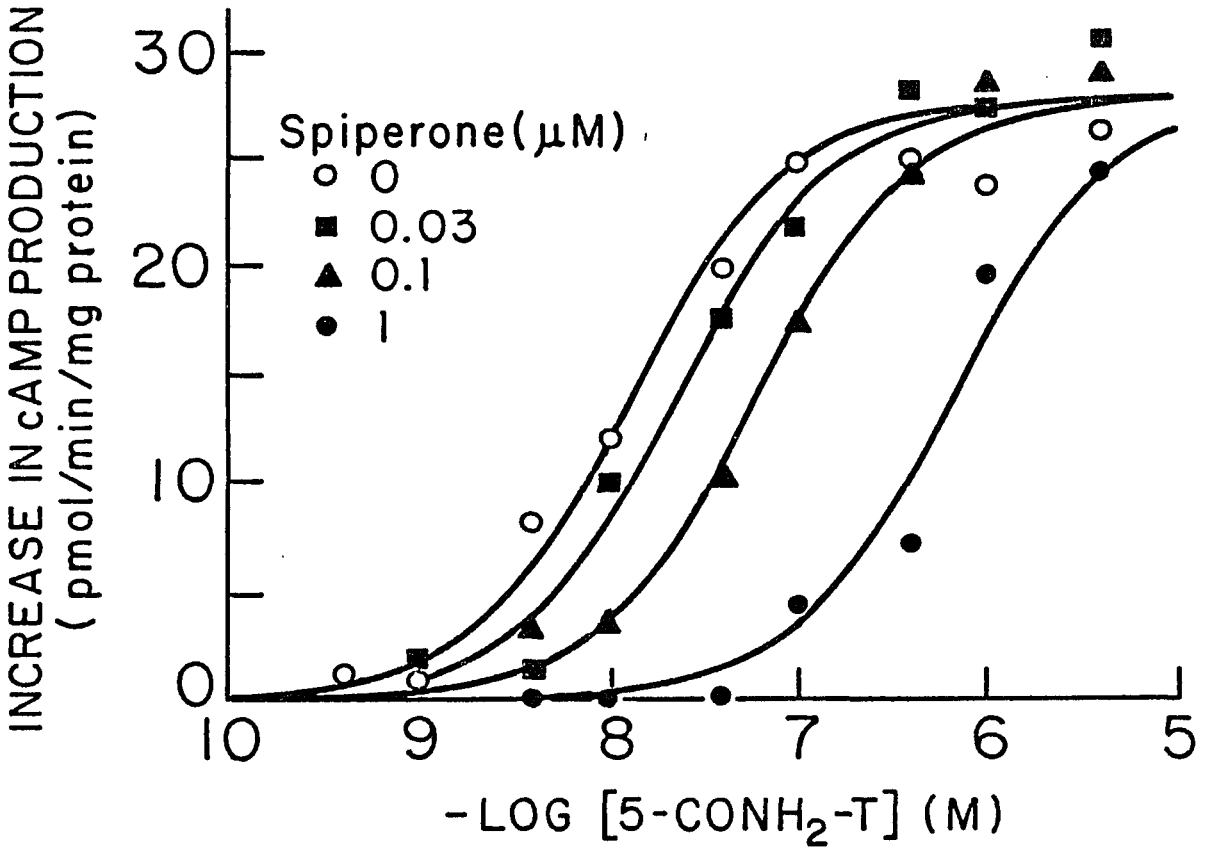

38.
30.
20.
10.
0.
Increase in cAMP Production
(pmol/min/mg protein)
.001
.01
.1
1.
10.
100.
[BUFOTENINE] (uM)

Fig. 16 Antagonism of 5-$CONH_2$-T by spiperone

Stimulation of adenylate cyclase activity by 5-$CONH_2$-T alone (O) and by 5-$CONH_2$-T in the presence of 30 nM (■), 100 nM (▲), and 1 uM (●) spiperone. Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 45.3 $\pm$ 0.08 pmol cAMP/min/mg protein. The curves are the best fit to the common slope model described in Methods (slope index = 1.05). The data were not fit significantly better by the variable slope model ($F_{(3,25)} = 1.48$). The degree of shift produced by each concentration of spiperone was expressed as the ratio of EC50 values in the presence and absence of antagonist (concentration ratio, CR). The data from this experiment and four others were used to construct a Schild plot (Fig. 17).


INCREASE IN cAMP PRODUCTION
(pmol/min/mg protein)
30
20
10
0
Spiperone(μM)
0
0.03
0.1
1
10
9
8
7
6
5
-LOG [5-CONH2-T] (M)

constrained to slope = 1.0 was therefore used to estimate the dissociation constant of spiperone at the receptor (R_H) activated by low concentrations of 5-$CONH_2$-T (Fig. 17). The intercept (pA_2 = 7.62 $\pm$ 0.06) corresponds to a KB of 24 nM. This direct estimate of the affinity of spiperone for R_H is very close to the value obtained indirectly by computer-fitting the data on antagonism of 5-HT by spiperone.

Because of the low potency of 5-$CONH_2$-T in eliciting the second component of stimulation it is difficult to verify the fitted affinity of spiperone for R_L. The computer-generated estimate of KB_L from one experiment in which the full response to 5-$CONH_2$-T was measured in the presence of 40 uM spiperone (Fig. 18) was 13 uM. Despite the "uncertainty" in this estimate, this fit was significantly better than one in which spiperone was assumed to have no affinity for R_L. In two other experiments, however, 10 and 40 uM spiperone had no detectable effect on the second component of the 5-$CONH_2$-T curve.

E. Antagonism by Ketanserin

Ketanserin at a concentration of 100 nM, which is at least 50 times its KB for the 5-HT_2 receptor, had negligible effect (< two-fold shift) on the concentration-response curves for 5-HT (n = 2) and 5-$CONH_2$-T (n = 1). Higher concentrations of ketanserin (1 - 50 uM) shifted the curve of 5-$CONH_2$-T to the right in a surmountable manner, but also

Fig. 17 Schild plot of antagonism of 5-$CONH_2$-T by spiperone

Schild plot of the data on antagonism of 5-$CONH_2$-T (0.4 nM - 4 uM) by spiperone. The data are from five experiments, including the one shown in Fig. 16. The regression of log (CR-1) vs log [spiperone] results in a straight line with a slope (1.10 $\pm$ 0.08) not significantly different from 1.0. The intercept of a line constrained to slope = 1.0 (shown here) was used to estimate the dissociation constant of spiperone at the receptor selectively activated by low concentrations of 5-$CONH_2$-T (i.e., R_H). The intercept (pA_2 = 7.62 $\pm$ 0.06) corresponds to a dissociation constant of 24 nM.

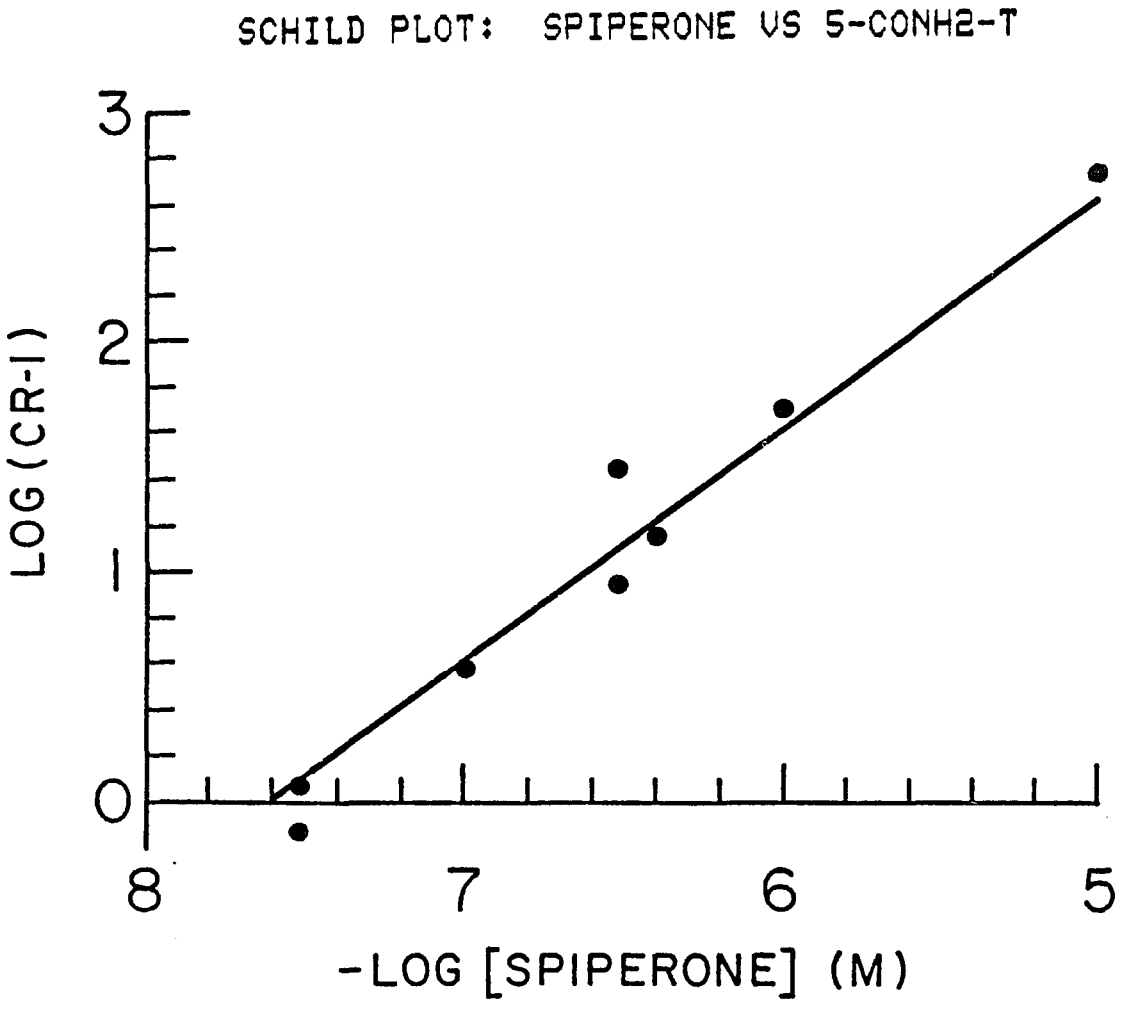
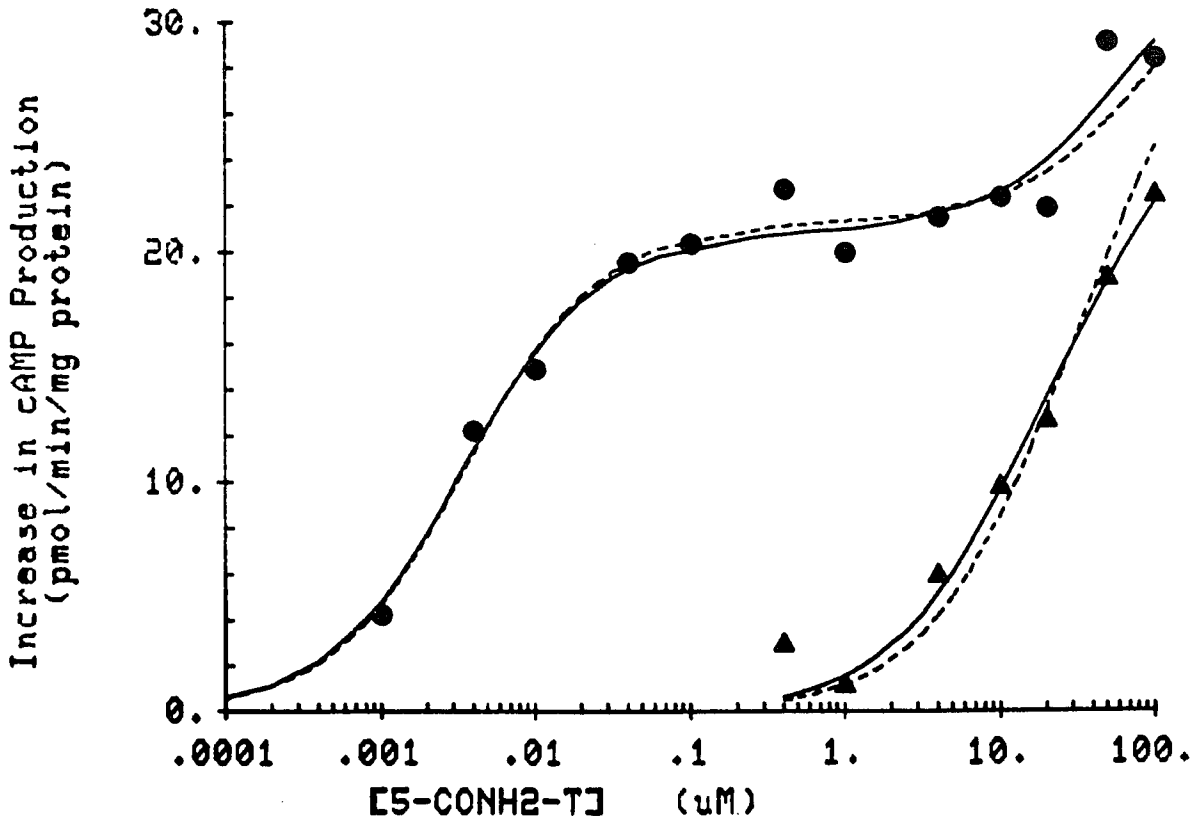

SCHILD PLOT: SPIPERONE VS 5-CONH2-T
LOG (CR-1)
3
2
1
0
8
7
6
5
-LOG [SPIPERONE] (M)

Fig. 18 Antagonism of 5-$CONH_2$-T by 40 uM spiperone

Stimulation of adenylate cyclase activity by 5-$CONH_2$-T alone (●) and by 5-$CONH_2$-T in the presence of 40 uM spiperone (▲). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 48.8 $\pm$ 1.0 pmol cAMP/min/mg protein. The data are from the same experiment shown in Fig. 10. Spiperone alone caused a 19% decrease in basal activity in this experiment; the decreased basal value was used to calculate net stimulation by 5-$CONH_2$-T in the presence of spiperone. The data were fit simultaneously to two functions similar to equation (3); on the basis of data with 5-HT, maximal response due to R_H plus R_L was set to 34.7 pmol cAMP/min/mg protein. The fit (solid lines) provided the following parameter estimates: proportion of maximal response due to R_H = 60%; for 5-$CONH_2$-T: K_H = 3 nM, K_L = 69 uM; for spiperone: KB_H = 10 nM, KB_L = 13 uM. This fit, which included KB_L as a variable parameter, provided a significantly better fit to the data ($F_{(1,14)}$ = 7.3) than a simpler model (fit indicated by dashed line) in which it was assumed that spiperone had no affinity for R_L.


Increase in cAMP Production
(pmol/min/mg protein)
30.
20.
10.
0.
.0001
.001
.01
.1
1.
10.
100.
[5-CONH2-T]
(uM)

caused a marked decrease in the slope of the curve (n = 4). Higher concentrations of ketanserin (4 - 100 uM) also shifted the 5-HT curve to the right, but in this case the shifts appeared parallel; it is unclear whether this effect is produced by ketanserin's complex inhibition of response mediated by R_H. Although preincubation of drugs and membranes for 8 min did not increase the magnitude of the shifts produced by ketanserin, under these conditions the maximal response to 5-HT was potentiated in the presence of 20 uM ketanserin (n = 2; +30%, +76%). These complex actions of ketanserin are not well understood; they occur at concentrations much greater than those needed to block 5-HT_2 receptors.

F. Effects of 5-HT_3 Antagonists

The peripheral neuronal 5-HT antagonists (-)cocaine and MDL 72222, at concentrations as high as 40 uM and 10 uM, respectively, were inactive alone and in combination with 5-HT (4 nM - 40 uM). Each drug was tested in two separate experiments.

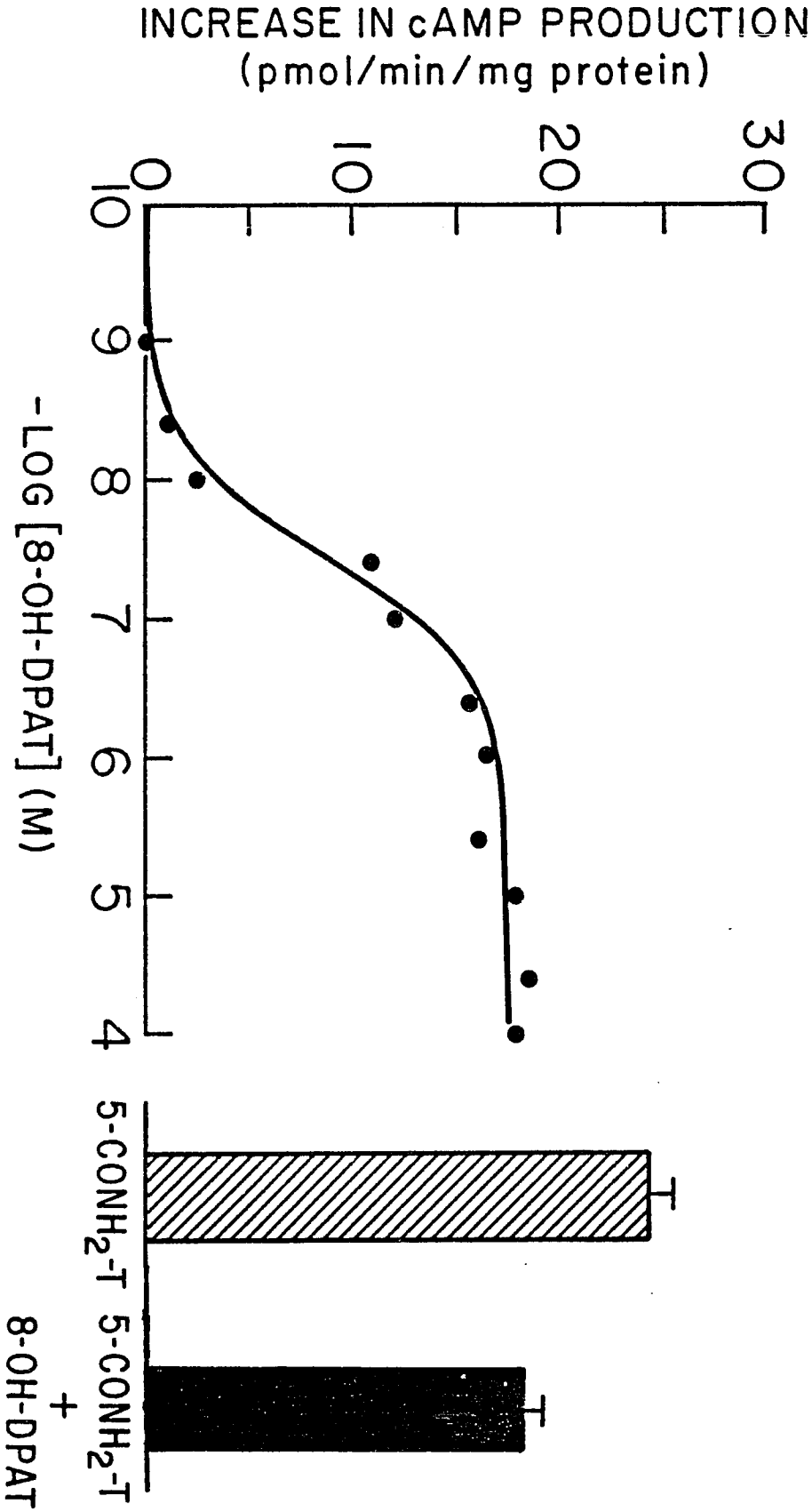
G. Drugs With Central Serotonergic Effects

(1) 8-OH-DPAT

8-OH-DPAT stimulated adenylate cyclase activity in this preparation (Fig. 19) with a mean EC50 of 29 $\pm$ 11 nM and a slope index (0.88 $\pm$ 0.08) that was not significantly different from 1.0 (n = 4). Maximal stimulation by 8-OH-DPAT represented 0.8 $\pm$ 0.1 of the stimulation elicited

Fig. 19 Stimulation of adenylate cyclase activity by 8-OH-DPAT

Each point represents the mean increase in cAMP production from a single experiment in which basal activity was 48.0 ± 0.5 pmol cAMP/min/mg protein. The curve is the best fit to the logistic function: EC50 = 36 nM; maximal response = 17.2 pmol cAMP/min/mg protein; slope index = 1.09. In this experiment the maximal response to 8-OH-DPAT represents 71% of the response to 0.4 uM 5-$CONH_2$-T. The bars (mean stimulation ± SE) show inhibition of the response to 0.4 uM 5-$CONH_2$-T by 4 uM 8-OH-DPAT.


INCREASE IN cAMP PRODUCTION
(pmol/min/mg protein)
0
10
20
30
10
9
8
7
6
5
4
-LOG [8-OH-DPAT] (M)
5-CONH2-T
5-CONH2-T
+
8-OH-DPAT

by 0.4 uM 5-$CONH_2$-T; a maximally effective concentration of 8-OH-DPAT inhibited the stimulation by 0.4 uM 5-$CONH_2$-T (Fig. 19). Concentrations of 8-OH-DPAT as high as 400 uM did not elicit a second component of stimulation. Spiperone (0.1 - 10 uM) was a simple competitive antagonist of 8-OH-DPAT, with a pA_2 value of 7.69 $\pm$ 0.13 (Fig. 20). The corresponding dissociation constant, 21 nM, is comparable to the values obtained when 5-HT, bufotenine, or 5-$CONH_2$-T were used to activate R_H. These results indicate that 8-OH-DPAT is a potent partial agonist at R_H, with an intrinsic activity of 0.8.

Although 8-OH-DPAT did not exhibit agonism at R_L, the possibility that it was an antagonist at R_L was investigated. The upper part of the concentration-response curve to 5-HT was shifted to the right in the presence of 4 uM 8-OH-DPAT (Fig. 21), a result that was expected from the simulation shown in Fig. 9. The data shown in Fig. 21 were adequately fit by a two-receptor model in which 8-OH-DPAT was assumed to have no affinity for R_L. The shift can be explained solely by the partial agonism of 8-OH-DPAT at R_H. A more complex model which included the antagonist dissociation constant of 8-OH-DPAT at R_L as a variable parameter did not provide a significantly better fit of the data. Similar results were obtained in two other experiments in which response to 5-HT was measured in the presence of higher concentrations of 8-OH-DPAT (40 and 50

Fig. 20 Schild plot of the antagonism of 8-OH-DPAT by spiperone

Schild plot of the data on antagonism of 8-OH-DPAT by spiperone from three experiments. The regression of log (CR-1) vs log [spiperone] results in a straight line with a slope (0.83 $\pm$ 0.13) not significantly different from 1.0. The intercept of a line constrained to slope = 1.0 (shown here) was used to estimate the dissociation constant of spiperone at the receptor activated by 8-OH-DPAT. The intercept (pA_2 = 7.69 $\pm$ 0.13) corresponds to a dissociation constant of 21 nM.

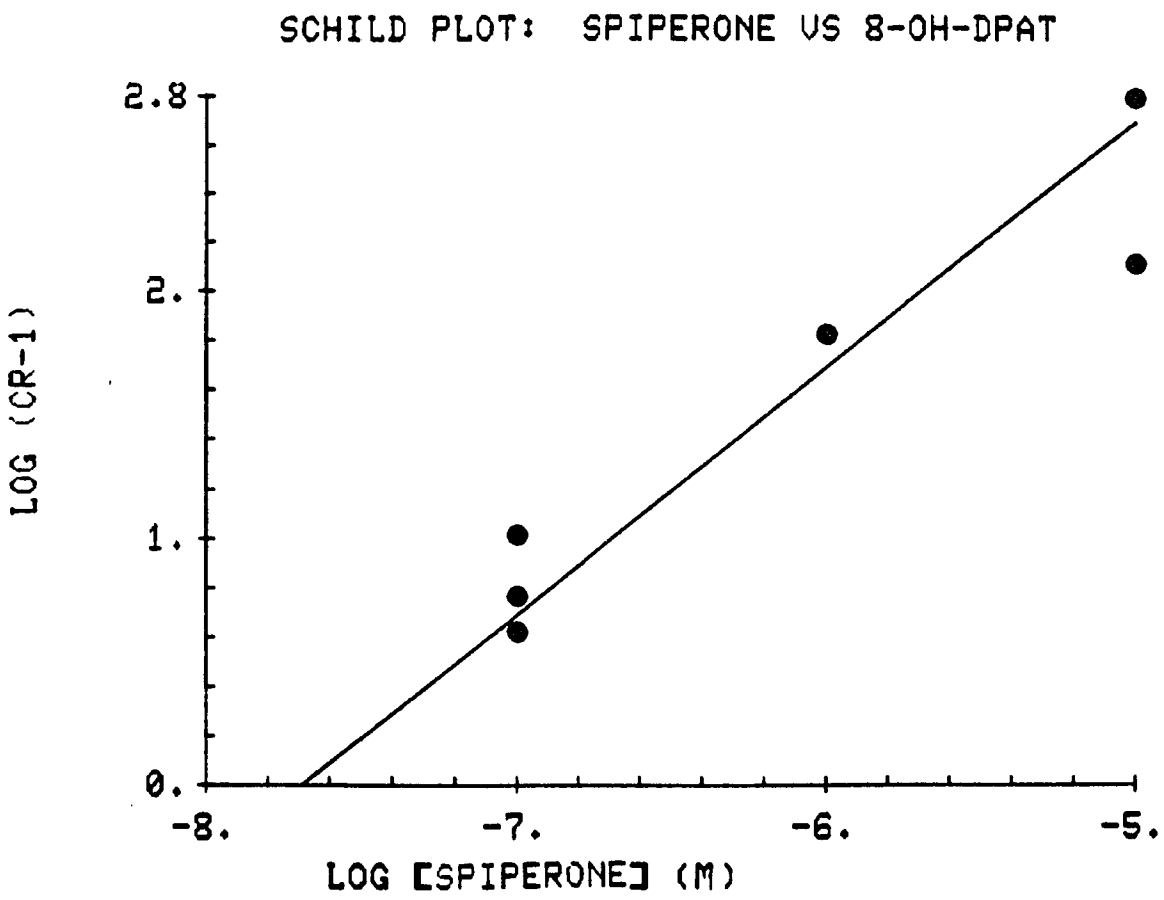
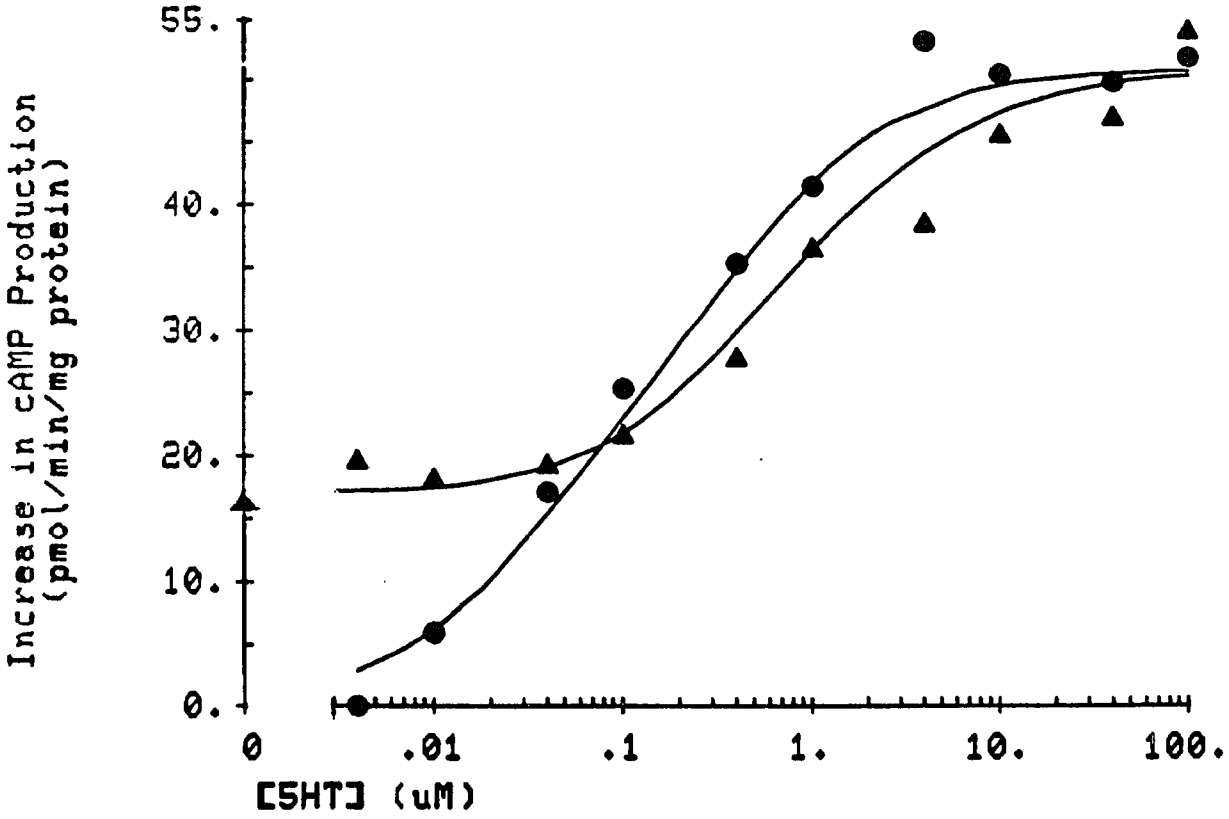

SCHILD PLOT: SPIPERONE VS 8-OH-DPAT
2.8
2.
1.
0.
LOG (CR-1)
-8.
-7.
-6.
-5.
LOG [SPIPERONE] (M)

Fig. 21 Stimulation of adenylate cyclase activity by 5-HT in the presence of 4 uM 8-OH-DPAT

Stimulation of adenylate cyclase activity by 5-HT alone (●) and by 5-HT in the presence of 4 uM 8-OH-DPAT (▲). Each point represents the mean increase in cAMP production from a single experiment in which basal enzyme activity was 48.0 ± 0.5 pmol cAMP/min/mg protein. The curves are the result of simultaneously fitting the response data for 5-HT alone and for 5-HT plus 8-OH-DPAT to equation (1) and equation (2), respectively. Stimulation by 0.4 uM 5-$CONH_2$-T (24.3 pmol cAMP/min/mg protein) was used as an estimate of $Emax_H$. The values of the following parameters were fixed on the basis of average results with 8-OH-DPAT: KB_H = 30 nM, β_H = 0.7, β_L = 0. In the fit shown here it was assumed that 4 uM 8-OH-DPAT had no antagonist activity at R_L ($KB_L = \infty$). This fit provided the following parameter estimates: $Emax_L$ = 26.2 pmol cAMP/min/mg protein; for 5-HT, K_H = 34 nM, K_L = 459 nM. A more complex model in which KB_L was included as a variable parameter did not provide a significantly better fit of the data ($F_{(1, 17)} = 3.08$).


Increase in cAMP Production
(pmol/min/mg protein)
55.
40.
30.
20.
10.
0.
0
.01
.1
1.
10.
100.
[5HT] (uM)

uM). At the concentrations tested, there is no evidence that 8-OH-DPAT is recognized at R_L.

(2) Buspirone

Buspirone stimulated adenylate cyclase activity in this preparation (Fig. 22). Although maximal stimulation elicited by buspirone was only 20 $\pm$ 1% over basal activity (n = 5), the concentration-response curve appeared monophasic; the EC50 value for buspirone was about 130 nM (n = 2). Maximal stimulation by buspirone represented 0.42 $\pm$ 0.03 of the stimulation elicited by 0.4 uM 5-$CONH_2$-T. In a pilot experiment, a high concentration of buspirone (40 uM) inhibited the response to 0.4 uM 5-$CONH_2$-T, suggesting that buspirone was a partial agonist at R_H. This hypothesis was tested by measuring the effect of buspirone on the concentration-response curve for 5-$CONH_2$-T (n = 3; Fig. 23). Buspirone (3 - 20 uM) shifted the 5-$CONH_2$-T curve to the right in a manner that was consistent with partial agonist activity at R_H. Computer-fits of these data provided an estimate of the dissociation constant of buspirone, 200 $\pm$ 92 nM (n = 3), that was close to its EC50 value. Concentrations of buspirone as high as 100 uM did not elicit a second component of stimulation. A high concentration of buspirone (50 uM) also appears ineffective in blocking the effect of 5-HT mediated by R_L (Shenker and Maayani, unpublished observations). These reslts indicate that buspirone is a potent partial agonist at R_H, with an intrinsic activity of 0.4.

Fig. 22 Stimulation of adenylate cyclase activity by buspirone

Stimulation of adenylate cyclase activity by buspirone (■) and by 5-HT (●). Each point represents the mean increase in cAMP accumulation from a single experiment in which basal activity was 37.2 ± 0.5 pmol cAMP/min/mg protein. Data is from same experiment shown in Fig. 8. The stimulation by 0.4 uM 5-$CONH_2$-T in this experiment was 17.7 pmol cAMP/min/mg protein. Fitting the buspirone data to the logistic equation provided the following parameter estimates: EC50 = 128 nM, slope index = 0.82, maximal response = 6.5 pmol cAMP/min/mg protein (0.37 of $Emax_H$).

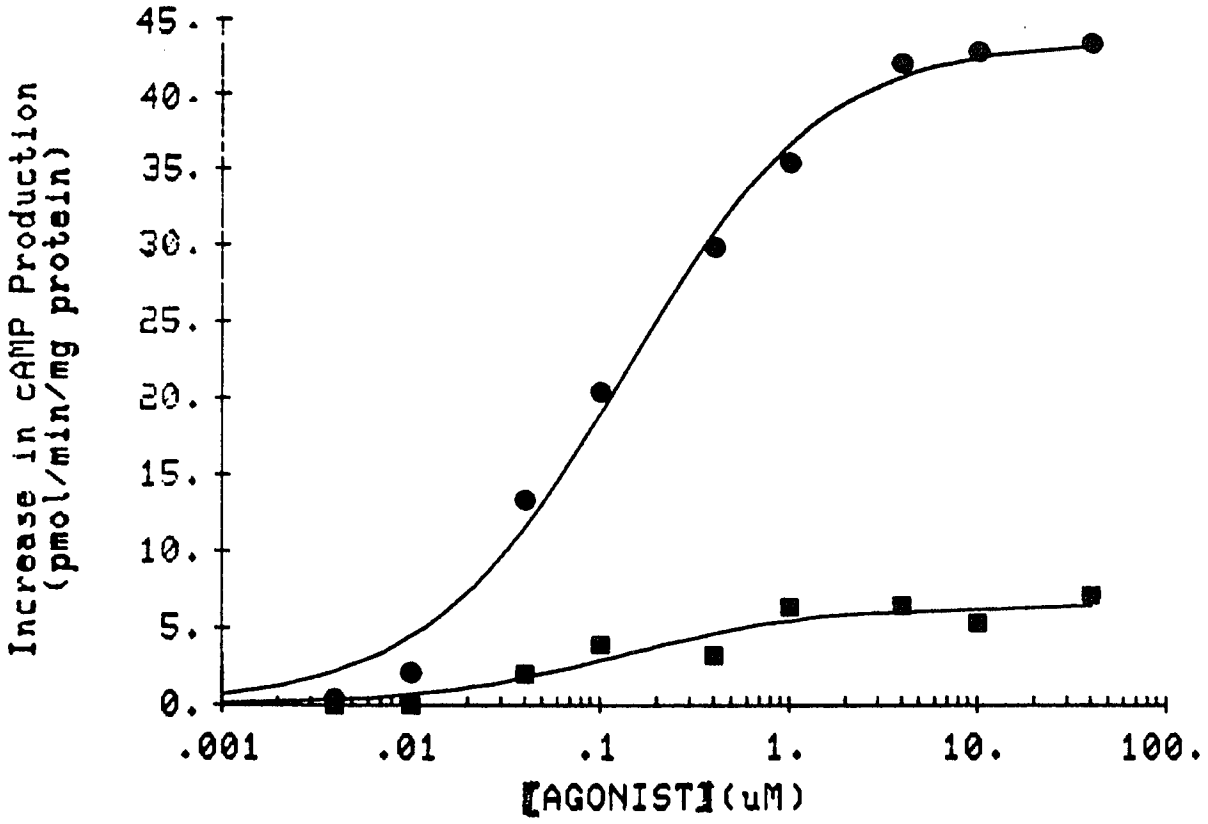
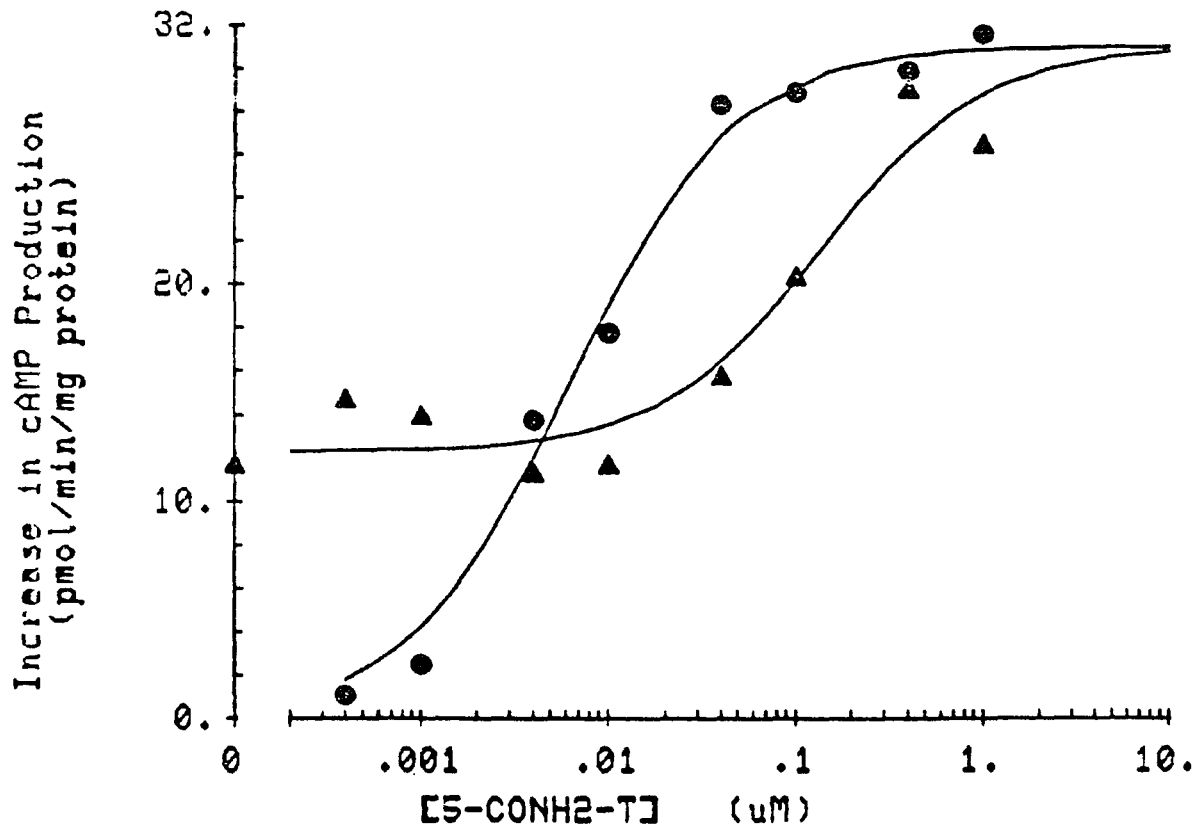

Increase in cAMP Production
(pmol/min/mg protein)
45.
40.
35.
30.
25.
20.
15.
10.
5.
0.
.001
.01
.1
1.
10.
100.
[AGONIST](uM)

Fig. 23 The effect of 3 uM buspirone on stimulation of adenylate cyclase activity by 5-$CONH_2$-T

Stimulation of adenylate cyclase activity by 5-$CONH_2$-T alone (●) and by 5-$CONH_2$-T in the presence of 3 uM buspirone (▲). Each point is the mean increase in cAMP accumulation from a single experiment in which basal enzyme activity was 52.8 ± 0.5 pmol cAMP/min/mg protein. These concentrations of 5-$CONH_2$-T are selective for R_H. Data were fit simultaneously to two simplified versions of equation (2) where $Emax_L = 0$, $\alpha_H = 1.0$, and [B] = 0 or 3 uM. The fit provided the following parameter estimates: $Emax_H$ = 30.9 pmol cAMP/min/mg protein; for 5-$CONH_2$-T, K_H = 6 nM; for buspirone, KB_H = 143 nM, B_H = 0.42.


Increase in cAMP Production
(pmol/min/mg protein)
32.
20.
10.
0.
0
.001
.01
.1
1.
10.
[5-CONH2-T]
(uM)

DISCUSSION

I. Initial Characterization of the Response to 5-HT

The modifications in assay conditions that were made in the first stage of this project allowed quantitative pharmacological characterization of 5-HT-stimulated adenylate cyclase activity in guinea pig hippocampal membranes. More rigorous studies on the effects of different membrane preparations and of different concentrations of assay components on adenylate cyclase activity could lead to conditions that provide even greater maximal stimulation by 5-HT. Because of the apparent desensitization of 5-HT receptors that occurs under the present conditions, a two minute assay was used routinely.

The stimulation of adenylate cyclase activity in the particulate fraction of adult guinea pig hippocampus by 5-HT was shown to be concentration-dependent, GTP-dependent, and additive to responses elicited by histamine, (-)isoproterenol, and dopamine, suggesting that distinct 5-HT receptors mediate the effect. Concentrations of 5-HT (and other drugs) greater than 100 uM appear to stimulate adenylate cyclase activity "nonspecifically" in this preparation. Effects of high concentrations of 5-HT that are not mediated by 5-HT receptors have previously been described (Humphrey, 1978). By using a limited concentration range, only responses mediated by 5-HT receptors can be studied.

The finding that concentrations of dopamine greater than 10 uM cross-react with 5-HT receptors in this preparation is not surprising. Similarity in the recognition sites of some dopamine and 5-HT receptors has previously been suggested (Enjalbert et al., 1978b; Weiss and Drummond, 1981), and the contractile effect of dopamine in canine arteries has clearly been shown to be due to activation of 5-HT receptors (Gilbert and Goldberg, 1975). After it became evident that stimulation by 5-HT in this preparation was mediated by two different 5-HT receptors, some additional additivity experiments were performed with 5-$CONH_2$-T and dopamine. The fact that responses to 0.4 uM 5-$CONH_2$-T and 100 uM dopamine were not strictly additive suggests that at least some, and possibly all, of the observed cross-reactivity was due to activation of R_H by high dopamine concentrations.

II. Alterations in 5-HT-Stimulated Cyclase Activity

The response to 5-HT, but not to histamine, is enhanced by treating guinea pigs with reserpine 24 hours before sacrifice. The findings that reserpine does not change basal activity, the EC50 value of 5-HT, and the slope index of the 5-HT concentration-response curve indicate that the increased response to 5-HT is not merely due to a reduction in the amount of endogenous 5-HT carried into the assay with the particulate preparation. It is unlikely that reserpine nonspecifically potentiates catalytic activity or

receptor-effector coupling efficacy because both basal and histamine-stimulated activities are unaltered by the treatment. Instead, the enhanced response to 5-HT may be due to an increase in 5-HT receptor concentration or an increase in the ability of the receptors to be activated by agonist.

A reasonable hypothesis is that a proportion of the 5-HT receptors in membranes prepared from untreated animals are in a "desensitized" state. These desensitized receptors would be incapable of stimulating adenylate cyclase because of their prior activation by 5-HT (e.g., Huidobro-Toro and Foree, 1980; Fillion et al., 1981). Decreasing 5-HT concentration in the vicinity of the receptors in vivo by treatment with reserpine may increase the ratio of functional to desensitized receptors in the membranes prepared for assay. Although total receptor number would not increase, the higher proportion of functional receptors in the membranes would be reflected by an increase in maximal stimulation by 5-HT.

Single injection with a reserpine-like compound, syrosingopine, results in increased maximal response to 5-HT in saphenous vein within 24 hours (Humphrey, 1978). Reserpinization has also been shown to increase stimulation of brain cAMP production by catecholamines: effects were reported within 24 hours (Trabucchi et al., 1976; Nahorski, 1977), and after longer periods of treatment (2 to 4 days)

(Ahn and Makman, 1977; Dolphin et al., 1979, and references therein).

Reserpine has been shown to cause a prolonged depression of firing of serotonergic neurons in the dorsal raphe of the rat (Baraban et al., 1978). A similar action in guinea pig raphe might contribute to the development of "supersensitivity" to 5-HT. Other treatments that deplete brain 5-HT (lesioning of serotonergic neurons, inhibition of 5-HT synthesis) have been shown to increase the maximal stimulation of adenylate cyclase activity by 5-HT in infant and adult rat brain (Hamon et al., 1981; Barbaccia et al., 1983).

After the reserpine experiments were completed, it became clear that there were actually two types of cyclase-linked 5-HT receptor in this preparation. The fact that reserpinization did not lead to a change in the EC50 value or slope index of the 5-HT concentration-response curve suggests that the proportion of total response mediated by R_H and R_L (about 50:50) did not change significantly, that is, responses mediated by both receptors were enhanced to approximately the same degree. More detailed studies with R_H-selective agonists such as 5-$CONH_2$-T and 8-OH-DPAT are required to establish whether endogenous 5-HT might normally exert a desensitizing effect on both R_H and R_L *in vivo*.

Evidence of desensitization of 5-HT receptors was obtained *in vitro*. The decrease in responsiveness to 5-HT

was 5-HT-dependent, indicating that the phenomenon was not merely due to degradation of adenylate cyclase components and/or receptors. When membranes were preincubated with the same range of 5-HT concentrations usually used to elicit stimulation, the decrease in maximal stimulation by 5-HT was shown to be concentration-dependent. This is consistent with the idea that loss of responsiveness occurs as a consequence of 5-HT receptor occupation. Loss of responsiveness to 5-HT in vitro must also be considered in light of the subsequent discovery of 5-HT receptor heterogeneity in this preparation; it is presently unclear whether both R_H or R_L can undergo desensitization. Future studies that employ selective agonists, or a brain region containing a homogeneous population of one of the receptors, could address this question.

Aside from Fillion and co-workers (Fillion et al., 1980b, 1981; Fillion, 1983), no one has previously presented evidence that desensitization of neurotransmitter-stimulated adenylate cyclase occurs in a broken-cell preparation of mammalian brain. Even among well-studied peripheral receptors coupled to adenylate cyclase, only a few have been reported to undergo desensitization in a cell-free preparation (Bockaert et al., 1976; Anderson and Jaworski, 1979; Ezra and Salomon, 1981; Nambi et al., 1984). Future studies of the alterations in 5-HT-stimulated adenylate cyclase activity described here may yield insights into the mechanism of desensitization in

general and the regulation of brain 5-HT receptors in particular.

III. The Two Receptor Model

Characterization of 5-HT-stimulated adenylate cyclase activity in guinea pig hippocampal membranes with selected agonists and the antagonist spiperone reveals the presence of two, pharmacologically distinct 5-HT receptors that mediate the same response. The presence of two receptors was investigated with the aid of computerized curve-fitting procedures. The findings that first suggested receptor heterogeneity included: 1) the shallow, non-parallel concentration-response curves for 5-HT, 5-MeOT, bufotenine and tryptamine, 2) the biphasic concentration-response curve for 5-$CONH_2$-T, and 3) the atypical antagonism of 5-HT by spiperone. Strong additional evidence for the two-receptor model was obtained from experiments in which the response to one agonist was measured in the presence of a selected concentration of another agonist. It was possible to determine the dissociation constant of spiperone for one of the receptors, R_H, with confidence; the value was the same when it was obtained "indirectly" by analyzing the complex antagonism of agonists that were only slightly selective for R_H (5-HT, bufotenine) and when it was obtained by analyzing the simple, competitive antagonism of agonists that were highly selective for R_H (5-$CONH_2$-T, 8-OH-DPAT). All the drugs studied thus far exhibit some selectivity for R_H.

Other systems have been described in which two populations of receptors mediating the same response are revealed by non-parallel concentration-response curves for agonists and/or by the complex behavior of antagonists; an especially relevant example is the description of a mixed population of $beta_1$- and $beta_2$-adrenergic receptors positively coupled to adenylate cyclase in glial cultures (Ebersolt et al., 1981b). Changes in the slope of an agonist curve in the presence of a selective antagonist have been simulated (Hough et al., 1980) and observed (Palacios et al., 1978; Hedberg and Mattson, 1981; Ebersolt et al., 1981b; O'Donnell and Wanstall, 1983; Johannson and Persson, 1983; Kaumann et al., 1983; Zaagsma et al., 1983). Several previous reports have focused on the fact that differential blockade of two receptors activated by a single agonist results in a Schild plot with a shallow slope, or one with an intercept that reflects an "amalgam" of the affinities of the antagonist for the two receptors (Furchgott, 1978; O'Donnell and Wanstall, 1979, 1981, 1983; Kenakin, 1982; Lemoine and Kaumann, 1983). As mentioned in Results, the slope of a Schild plot constucted from the "common slope" fit of data on antagonism of 5-HT by spiperone is indeed shallow; furthermore, the intercept of that regression (7.3) represents a value between the affinities of spiperone for R_H and R_L.

The advantages of computerized curve-fitting for the accurate resolution of receptor subtypes have been

well-documented in the case of radioligand binding data (DeLean et al., 1982; Burgisser, 1983), but this method has not often been applied to studies of functional receptors. Fitting response data directly to equations based on the two-receptor model proved satisfactory, suggesting that this approach may be useful in analyzing other systems where heterogeneity of functional receptors is suspected.

Regardless of whether graphical or computer-modeling methods are used, deviations from a one-receptor model may be too subtle to detect experimentally unless highly selective agonists or antagonists are examined (Hough et al., 1980; Lemoine and Kaumann, 1983; O'Donnell and Wanstall, 1983; Zaagsma et al., 1983). In addition to the selectivity of the drugs used, the confidence with which one is able to demonstrate the presence of two receptors depends on the ratio of $Emax_H/Emax_L$, the number of data points collected, the scatter of the data points, and the concentration range of drug studied (see Hough et al., 1980; DeLean, et al., 1982; Burgisser, 1983). For example, fitted slope indices for the concentration- response curves for 5-HT, an agonist which is only about 10-fold selective for R_H, ranged from 0.59 to 1.15 in these studies. The complex antagonism of 5-HT by spiperone, which is at least 100-fold selective for R_H, might have been overlooked if fewer drug concentrations were used, or if the scatter in the data had been somewhat greater.

The availability of a highly selective agonist is critical for the unambiguous characterization of one receptor type in a mixed population. While 5-$CONH_2$-T and 8-OH-DPAT are available for the characterization of R_H , the lack of drugs selective for R_L make it difficult to characterize that receptor in greater detail in this preparation. Identifying preparations that contain only R_H or R_L would allow each receptor to be characterized independently and provide additional verification of the two-receptor model.

The initial characterization of agonist potency at R_H and R_L that is presented here (Table 7) may provide a basis for future structure-activity studies. 5-HT and 5-MeOT are esentially equipotent and exhibit 10-fold selectivity for R_H. Dimethylation of the side chain amine of 5-HT (to form bufotenine) or removal of the 5-hydroxyl group (to form tryptamine) reduces the apparent affinity for R_L proportionately more than for R_H, resulting in compounds that have 50-fold selectivity for R_H. Dimethylation may also be associated with a reduction in intrinsic activity at R_L. Most striking is the fact that the single substitution of the carboxamido group for the hydroxyl group of 5-HT produces an analog with 7-fold higher affinity for R_H but 80-fold lower affinity for R_L. Two compounds with very different structures, 8-OH-DPAT and buspirone, are potent partial agonists at R_H but are apparently inactive at R_L; studying analogs of these two drugs may help identify the

structural characteristics that are associated with their selectivity and with their reduced intrinsic activity at R_H.

IV. Classification of R_H and R_L

A. Relation to 5-HT_2 and 5-HT_3 Receptors

The apparent dissociation constants of spiperone for R_H (20 nM) and R_L ($\geq$ 2 uM) are much higher than the dissociation constant of spiperone for the 5-HT_2 receptor (1 nM) (Humphrey et al., 1982). To confirm the fact that neither of the cyclase-linked receptors corresponds to the 5-HT_2 receptor, the activity of ketanserin was evaluated. Ketanserin at a concentration of 100 nM, which is at least 50 times its dissociation constant at the 5-HT_2 receptor (Van Nueten et al., 1981; Leysen et al., 1982; Humphrey et al., 1982), had negligible effect (< two-fold shift) on the concentration-response curves for 5-HT and 5-$CONH_2$-T. These data show that neither R_H nor R_L may be classified as a 5-HT_2 receptor, an inference consistent with previous reports that 5-HT receptors coupled to adenylate cyclase are not 5-HT_2-like (Peroutka et al., 1981; Barbaccia et al., 1983).

The 5-HT_3 antagonists (-)cocaine (K_B = 1 uM; Fozard et al., 1979) and MDL 72222 (IC50 = 1 nM; Fozard, 1984) were inactive in this system at concentrations of 40 uM and 10 uM, respectively. In addition, 5-MeOT is apparently equipotent with 5-HT at both cyclase-linked receptors,

whereas it is much less potent than 5-HT at 5-HT_3 receptors (Fozard and Mobarok Ali, 1978; Fozard, 1983a; Keele and Armstrong, 1964). Thus, neither R_H nor R_L may be classified as a 5-HT_3 receptor.

Since micromolar concentrations of (-)cocaine inhibit 5-HT-mediated contraction of methysergide-treated guinea pig ileum (Fozard et al., 1979), it is also unlikely that either cyclase-coupled 5-HT receptor corresponds to the neuronal "M" receptor described by Gaddum and Picarelli (1957).

B. Relation to Other Cyclase-Linked 5-HT Receptors

Comparison of the two 5-HT receptors in guinea pig hippocampal membranes with the cyclase-coupled 5-HT receptors previously described in invertebrates, mammalian peripheral tissues, and mammalian brain is difficult, as the pharmacological characteristics of most of these systems are not known in detail. As mentioned in the Introduction, the existence of two central cyclase-linked 5-HT receptors, one neuronal and one glial, has been reported by Fillion and co-workers (Fillion et al., 1983), but not corroborated (Enjalbert et al., 1978a; Nelson et al., 1980b; Ebersolt et al., 1981a; Premont et al., 1983; Chneiweiss et al., 1984). Insufficient pharmacological data precludes relating the two receptors described by Fillion to the two in guinea pig hippocampus. Brain regions other than hippocampus, or other systems where 5-HT-stimulated cyclase activity has been measured, may contain different proportions of R_H and R_L;

such regional differences have been shown for cyclase-linked $beta_1$- and $beta_2$-adrenergic receptors (Dolphin et al., 1979). The micromolar EC50 of 5-HT found in most other cyclase systems resembles the estimated affinity of 5-HT for R_L in this system. The fact that spiperone is a weak inhibitor (micromolar IC50 value) of 5-HT-stimulated adenylate cyclase activity in cultured NCB-20 cells (Berry-Kravis and Dawson, 1983), in liver fluke (McNall and Mansour, 1984), and in infant rat colliculi (Nelson et al., 1979, 1980a) also suggests that R_L plays a significant role in those systems.

The results of experiments recently performed in this laboratory provide support for the hypothesis that stimulation of cyclase activity by 5-HT in infant rat colliculi is mediated predominantly by R_L. Quipazine, TFMPP, and RU 24969 are inactive at cyclase-coupled 5-HT receptors in infant rat colliculi (Nelson et al., 1980a; Euvrard and Boissier, 1980). All these compounds stimulate adenylate cyclase activity in guinea pig hippocampal membranes by activating R_H, but they appear to have little or no activity at R_L. RU 28253, an isomer of RU 24969 which is an agonist at collicular 5-HT receptors (Hunt et al., 1981), is also an agonist at R_L in guinea pig hippocampal membranes (Shenker and Maayani, unpublished observations). Although 8-OH-DPAT appears inactive at R_L, it was recently described as being a weak agonist (EC50 = 9 uM) at the collicular receptors (Hamon et al., 1984b). Additional

studies are needed to establish whether R_L and the 5-HT receptors mediating stimulation of adenylate cyclase activity in infant rat colliculi are indeed homologous.

C. Relation to 5-HT_1 Binding Sites

As discussed in the Introduction, the relationship between 5-HT receptors coupled to adenylate cyclase and binding sites labeled by ^{3}H-5-HT in mammalian brain is controversial. Since it is now clear that neither population is homogeneous, the relationship requires careful reevaluation. Results from this study suggest that R_H is the functional correlate of the 5-HT_{1A} binding site:

1) R_H has been defined in guinea pig hippocampus, a region known to contain a high density of 5-HT_{1A} binding sites (Schnellmann et al., 1984).

2) The dissociation constants of spiperone for R_H (20 nM) and for the 5-HT_{1A} binding site in guinea pig hippocampus (15 nM) (Schnellmann et al., 1984) are indistinguishable.

3) The 5-HT_{1A}-selective agonist 8-OH-DPAT is approximately equipotent with 5-HT in binding to 5-HT_{1A} sites (Middlemiss and Fozard, 1983; Hamon et al., 1984b) and in eliciting stimulation through R_H.

4) 5-$CONH_2$-T is several times more potent than 5-HT in binding to 5-HT_{1A} sites (Engel et al., 1983; Ebersole and Maayani, unpublished observations) and in eliciting stimulation through R_H.

5) Buspirone is several times less potent than 5-HT in binding to 5-HT_{1A} sites (Gozlan et al., 1983; Ebersole and Maayani, unpublished observations) and in eliciting stimulation through R_H.

6) Both R_H and the 5-HT_{1A} site are relatively insensitive to ketanserin (Awouters et al., 1982; Engel et al., 1983; Gozlan et al., 1983).

6) The apparent affinity of an agonist (e.g. 5-HT, 8-OH-DPAT) for the 5-HT_{1A} site defined in a standard binding assay lacking GTP is about 10-fold higher than its apparent affinity for R_H measured in the cyclase assay containing GTP. Based on what is known about the GTP-sensitivity of high-affinity agonist binding states of other receptors coupled to adenylate cyclase (e.g. Birnbaumer and Iyengar, 1982), this difference is expected. The affinity of agonists for 5-HT_1 sites, including those in hippocampus, has been shown to be reduced by guanine nucleotides (Peroutka et al., 1979; Mallat and Hamon, 1982; Sills et al., 1984).

If R_H indeed represents the functional correlate of the 5-HT_{1A} binding site then it is reasonable to suggest that in the future it be referred to as the 5-HT_{1A} <u>receptor</u>.

Although the low apparent affinity of spiperone and 8-OH-DPAT for R_L could suggest that this receptor corresponds to the 5-HT_{1B} binding site, other evidence is not consistent with this hypothesis: 5-HT, tryptamine, and 5-$CONH_2$-T also discriminate between R_H and R_L, yet none of

them appears to discriminate between the 5-HT_{1A} and 5-HT_{1B} binding sites (Pedigo et al., 1981; Engel et al., 1983; Ebersole and Maayani, unpublished observations).

The identification of drugs selective for the 5-HT_{1B} site might help define its functional correlate. Recent work suggests that the 5-HT_{1B} site is related to the presynaptic 5-HT autoreceptor in rat cortex (Engel et al., 1983; Middlemiss, 1984a, b). They resemble each other pharmacologically in their low apparent affinity for spiperone (Pedigo et al., 1981; Baumann and Waldmeier, 1981) and for 8-OH-DPAT (Middlemiss and Fozard, 1983; Middlemiss, 1984a) and in their high affinity for 5-$CONH_2$-T (Engel et al., 1983). A preliminary report indicates that 5-HT autoreceptor function is not mediated by cAMP accumulation (Schlicker et al., 1984), a finding that is consistent with the classification scheme discussed here.

D. Relation to 5-HT_1-like Receptors in the Periphery

As mentioned in the Introduction, 5-$CONH_2$-T is a highly potent agonist at several peripheral 5-HT receptors classified as "atypical" by virtue of their relative insensitivity to ketanserin and/or classical 5-HT antagonists (see Table 3 for references). 5-$CONH_2$-T is a potent agonist at the postsynaptic 5-HT receptor in canine saphenous vein, but spiperone is a relatively weak antagonist there (dissociation constant > 0.1 uM) (Feniuk et al., 1983a). This receptor could tentatively be referred to

as 5-HT_{1B}-like. The affinities of spiperone for other 5-HT receptors where 5-CONH_2-T appears more potent than 5-HT (the presynaptic receptor in canine saphenous vein, the receptors which mediate relaxation of cat saphenous vein and guinea pig ileum, the atypical receptors of the porcine carotid bed) have not been reported. It will be especially interesting to see if any of the atypical 5-HT receptors that mediate relaxation of smooth muscle correspond to R_H, as cAMP has been implicated in this process (Trevethick et al., 1984).

Spiperone and 8-OH-DPAT have already proven useful in classifying different types of 5-HT_1 binding sites and 5-HT_1-like receptors in the brain. They may also be helpful in discriminating types of atypical 5-HT receptors in the periphery, although the high affinity of spiperone for other receptor types (Awouters et al., 1982) may limit its utility. Establishing a definitive taxonomy of 5-HT receptors clearly requires the development of new antagonists selective for atypical 5-HT receptors.

V. Physiological Significance

Attributing a physiological effect to a receptor-mediated increase in cAMP production requires comparison of the pharmacological profiles of the physiological and the biochemical responses. Thus far, the biochemical, physiological and behavioral consequences of 5-HT-stimulated cAMP production have been most thoroughly

studied in simpler invertebrate systems (Lingle et al., 1982). In peripheral mammalian preparations, 5-HT-stimulated increases in cAMP have been implicated in increased chloride secretion by corneal epithelium (Klyce et al., 1982), regulation of skeletal muscle metabolism (Garber, 1977; Ezrailson et al., 1983), alterations in renal function (Shah et al., 1979), synaptic excitation of myenteric neurons (Nemeth et al., 1984), and relaxation of smooth muscle (Trevethick et al., 1984).

Defining the neurophysiological and behavioral consequences of 5-HT-stimulated adenylate cyclase activity mediated by R_H and R_L in mammalian brain depends on additional pharmacological studies, as well as a description of the anatomical distribution of R_H- and R_L-mediated activity. 5-HT-stimulated adenylate cyclase activity has also been measured in hippocampus of adult rat (Barbaccia et al., 1983; Shenker et al., 1983b) and data from this laboratory suggest that the activity is mediated by both R_H and R_L (Shenker and Maayani, unpublished observations). In several regions of rat CNS, including hippocampus, the results of lesioning studies suggest a postsynaptic neuronal localization of 5-HT-sensitive cyclase activity (Enjalbert et al., 1978a; Fillion et al., 1979a; Nelson et al., 1980b; Hamon et al., 1981; Barbaccia et al., 1983). Future attempts to relate the $5\text{-}HT_{1A}$ binding site to R_H should consider the finding that $5\text{-}HT_{1A}$ sites labeled by ^3H-8-OH-DPAT in rat hippocampus are also located on

postsynaptic neurons (Gozlan et al., 1983). As discussed previously, the question whether some cyclase-linked 5-HT receptors in rodent brain are located on glial cells is unresolved.

Several central serotonergic effects may involve cAMP as a second messenger:

1) 5-HT-stimulated phosphorylation of synapsin I

5-HT-stimulated phosphorylation of synapsin I in facial nucleus may be mediated by presynaptic 5-HT receptors coupled to adenylate cyclase (Dolphin and Greengard, 1981). These receptors are suggested to exist on the terminals of non-serotonergic neurons; their activation may lead to facilitation of neurotransmitter release (Nestler and Greengard, 1983). Little is known about the pharmacological properties of these receptors.

2) Serotonin Behavioral Syndrome

Features of the "serotonin behavioral syndrome" produced in rats by 5-methoxy-N,N-dimethyltryptamine and 8-OH-DPAT are blocked by spiperone and certain other antagonists, but not by ketanserin (Jacobs, 1974; Lucki et al., 1984; Tricklebank, 1984). These and other findings have led to the hypothesis that activation of 5-HT_{1A}-like receptors in the brain stem or spinal cord contributes to expression of the syndrome (Lucki et al., 1984; Tricklebank, 1984). The pharmacological properties of the receptors that mediate the behavioral syndrome are very similar to the properties of R_H described here. Preliminary experiments

indicate that drugs other than 8-OH-DPAT and buspirone which induce the behavioral syndrome (dimethyltryptamine, 5-methoxy-N,N-dimethyltryptamine, LSD, lisuride) (Silbergeld and Hruska, 1979; Sloviter et al., 1980; Lucki et al., 1984) also share the ability to activate R_H in the hippocampal cyclase system (Shenker and Maayani, unpublished results). It is conceivable that the lack of consensus regarding the efficacy of buspirone in eliciting the syndrome (Hjorth and Carlsson, 1982; McMillen and Mattiace, 1983; Eison et al., 1983) is related to the low intrinsic activity of buspirone at R_H. The possibility that a cyclase-linked 5-HT_{1A} receptor (i.e., R_H) is involved in expression of the serotonin behavioral syndrome would be strengthened by the demonstration of R_H-mediated stimulation of cyclase activity in the brain stem or spinal cord of rat.

Two other serotonergic behavioral effects that have been attributed to the activation of 5-HT_1-like receptors are guinea pig myoclonus (Luscombe et al., 1984) and rotational behavior in rats with unilateral raphe lesions (Blackburn et al., 1984); 8-OH-DPAT is potent in eliciting the rotational behavior. Additional work is needed to determine whether these responses are related to R_H.

3) Electrophysiological Responses

Different electrophysiological effects of 5-HT in mammalian brain regions, including hippocampus, have been attributed to increased or decreased potassium conductance (Segal, 1980; Aghajanian and Lakoski, 1984); it is possible

that modulation of potassium channels in brain is mediated by 5-HT-stimulated accumulation of cAMP, as has been shown in invertebrate neuronal systems (Lingle et al., 1982).

R_H and the 5-HT autoreceptor that mediates depression of firing by raphe serotonergic neurons are both relatively insensitive to blockade by ketanserin (Heym et al., 1984; Lakoski and Aghajanian, 1984); the affinity of the raphe autoreceptor for spiperone has not been reported. In respect to agonists, the receptors have been found to resemble each other (Shenker and Maayani, unpublished observations): a group of structurally diverse compounds (8-OH-DPAT, buspirone, LSD, lisuride, 5-HT, dimethyltryptamine, and 5-methoxy-N,N-dimethyltryptamine) share the ability to depress raphe firing (Aghajanian, 1981; Hjorth et al., 1982; Fallon et al., 1983; VanderMaelen et al., 1984) and to activate R_H. The recently-introduced raphe slice preparation (VanderMaelen et al., 1984; Aghajanian and Lakoski, 1984) may yield more quantitative data on the pharmacological characteristics of the autoreceptor.

The 5-HT-mimetic activity of buspirone at R_H and at the raphe autoreceptor may be important in understanding its anxiolytic action. The EC50 of buspirone for eliciting stimulation through R_H (about 130 nM) is comparable to the concentrations of buspirone which inhibit firing of serotonergic raphe neurons (100-400 nM) (VanderMaelen and Wilderman, 1984). Production of anti-conflict effects in

animals is used as a predictor of antianxiety effects in man; drugs which produce anti-conflict effects, including benzodiazepines, buspirone, and the 5-HT synthesis inhibitor p-chlorphenylalanine, share the ability to reduce serotonergic neurotransmission (Stein et al., 1973; Soubrie et al., 1983; VanderMaelen and Wilderman, 1984). Interestingly, it was recently reported that 8-OH-DPAT also shows anti-conflict effects in rats (J. Engel et al., 1984).

VI. Conclusions

The initial pharmacological characterization of two different 5-HT receptors coupled to adenylate cyclase in mammalian hippocampus has been performed. Neither receptor may be classified as the 5-HT_2 or as the 5-HT_3 type. The receptor with relatively high affinity for 5-HT, $5\text{-CONH}_2\text{-T}$, 8-OH-DPAT, buspirone, and spiperone appears to be the functional correlate of the 5-HT_{1A} binding site and may mediate some of the behavioral and electrophysiological effects of 5-HT. The significance of the receptor with lower affinity for 5-HT is less clear; it probably does not correspond to a known 5-HT binding site, but it may be responsible for the stimulation of adenylate cyclase activity by 5-HT measured in other systems, including infant rat colliculi.

In addition to increasing our understanding of the CNS actions of 5-HT, the assay of 5-HT-stimulated adenylate

cyclase activity may serve as a system for the development of new serotonergic agonists and antagonists. New antagonists selective for the cyclase-coupled 5-HT receptors would be valuable not only in defining the physiological consequences of 5-HT-stimulated accumulation of cAMP and for definitive classification of multiple 5-HT receptors, but may also prove to possess therapeutic properties, as has been shown for the selective antagonists of other monoamine receptors (histamine H_2, beta$_1$-adrenergic, dopamine D_2) that regulate adenylate cyclase activity.

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