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ENDOGENOUS LIGANDS FOR HIGH-AFFINITY RECOGNITION SITES OF PSYCHOTROPIC DRUGS

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INTRODUCTION

Neuroscience is a relatively young discipline, with many frontiers to explore and much territory still uncharted. Although discoveries of new endogenous chemicals that participate in neuron-to-neuron signaling have cast some light on specific neuronal models, they have not always led to a more fundamental understanding of the molecular mechanisms at work in the brain's storage, retrieval, and elaboration of information.

Less than 20 years ago, after much first-class research, neuroscientists knew that a few amino acids and some amines functioned as neutotransmitters, and they guessed that others might be involved in cell-to-cell signaling. At present, at least 50 different chemical signals, many of them polypeptides, are known to operate at synapses; still more are under investigation. Some of these new neuromodulators were discovered during studies of centrally acting drugs. The discovery that some drugs act on high-affinity recognition sites of neurons prompted researchers to use those drugs as probes to detect endogenous molecules acting in neuronal communication within nervous tissues.

This review focuses on several neuromodulators—endogenous ligands for benzodiazepine, phencyclidine, imipramine, and ketanserin recognition sites—and how they can be used to enlarge our understanding of the molecular language of nerve cells. The underlying hope is that such knowledge will elucidate the biochemical basis of learning and memory, i.e. how new neuronal circuitry and brain molecular traces are laid down during elaboration and categorization of incoming information.

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PUTATIVE ENDOGENOUS ALLOSTERIC MODULATORS OF $GABA_A$ RECEPTORS

Role of Putative Endogenous Ligands for the Benzodiazepine (BZ) Recognition Sites

At least two categories of BZ recognition sites (1–3) exist in the central nervous system of mammals. One is spatially and functionally associated with the $GABA_A$ receptor (4–6), the other is unrelated to it (Table 1).

Synaptic membranes contain high-affinity recognition sites for BZ (1, 2), which can positively and negatively modulate GABA-mediated behavioral or biochemical responses depending on the ligand (4–7). Given evidence of polytypic signaling at many synapses, BZ recognition sites are likely to be the target for a chemical signal that coexists and in certain situations is coreleased with the inhibitory transmitter GABA. This signal would be designed to modulate the action of GABA allosterically (8, 9). In fact, in the CNS specific BZ recognition sites are found on the alpha subunits and the GABA recognition sites on the beta subunits of the same tetrameric protein structure that forms the $GABA_A$ Cl^- channel complex (6, 10, 11).

As summarized in Table 1, at least three homologous but pharmacologically distinct subtypes of $GABA_A$ receptors are believed to be functionally associated with the BZ recognition sites. Assuming that the BZ sites are always associated with alpha subunits, we do not yet know whether these receptor subtypes represent (*a*) heterologous complexes expressing genetically predetermined differences in alpha subunits with the beta subunits being constant, or (*b*) one complex that undergoes changes in conformation due to differences in glycosylation or other posttranslational modifications. However, it is clear that various ligands for the BZ recognition sites associated with each category of $GABA_A$ receptor (Table 1) elicit different types of pharmacologically predictable, GABA-mediated behavioral, electrophysiological, and biochemical responses. Thus, the postsynaptic binding sites for GABA that are linked to a Cl^- channel and contribute to forming the $GABA_A$ receptor may well be the convergence points for information conveyed by a variety of chemical signals (4, 5, 7, 8, 12).

GABA activates the transducer directly, opening specific anion channels and thus allowing Cl^- to flow across the neuronal plasma membrane in a direction dependent upon its concentration gradient (13–16). The effectors of BZ recognition sites may act as positive (increasing probability) or negative (decreasing probability) allosteric modulators of GABA's action, i.e. they prolong or shorten the duration of Cl^- channel opening elicited by GABA (13–16).

Pharmacological experiments with a large group of BZ ligands suggest that the BZ recognition sites have the capacity to change configuration when they interact with different ligands and, according to the molecular properties of

Table 1 Ligands for benzodiazepine recognition sites linked to various CNS receptors

Type of CNS receptor	Anatomical location	Selective ligands for the modulatory site		Modulator antagonist	Mechanism of allosteric action
		Positive	*Negative*		
$GABA_{A1}$	Neuronal membranes (cortex-cerebellum)	Cl-218, 872	β-carbolines	Flumazenil	GABA recognition
$GABA_{A2}$	Neuronal membranes (spinal cord)	Clonazepam	——	Flumazenil	GABA recognition
$GABA_{A3}$	Neuronal membranes (?)	Ro5-4864	——	PK11195	TBPS-GABA interaction
(?)	Mitochondrial membranes (glia)	Ro5-4864	——	PK11195	Anion transport?

the ligand that is bound, mediate a positive or negative modulation of the $GABA_A$ receptor (4, 7, 8, 12, 17, 18). This modulation of GABA transmission is responsible for many of the centrally mediated effects of BZ recognition sites, including convulsant, anticonvulsant, anxiolytic, and anxiogenic actions (4, 5, 7, 19–21). One class of ligands (anxiolytic, anticonvulsant BZ) increases the probability of Cl^- channel gating by GABA; another class (anxiogenic, proconvulsant beta-carbolines [BC]) decreases this probability. These two classes of ligands cannot, however, be termed agonists or inverse agonists (21) because they are inactive in the absence of the receptor agonist GABA (4, 5). Furthermore, they do not release GABA (4, 5, 17) or act directly on the GABA receptor by displacing GABA from its recognition sites (4, 5, 7, 12, 22, 23). Provisionally, these ligands could be referred to as positive (BZ) or negative (BC) "allosteric modulators" of the $GABA_A$ recognition sites. The imidazobenzodiazepine flumazenil also binds with high affinity to BZ recognition sites. Although it is devoid of intrinsic modulatory activity, when bound to the BZ site associated to GABA receptor it blocks the modulatory actions of BZ and BC (24).

As previously mentioned, there is another class of BZ binding sites in the CNS (revealed by radioreceptor binding studies) that is unrelated to the $GABA_A$ receptors (Table 1). These sites are found in glial cells and in some specialized neurons (25, 26) and are broadly distributed in many non-neuronal tissues, including adrenals (cortex but not medulla), testes, kidney, liver, and several tumor cell lines (25). These binding sites have provisionally been termed "peripheral" or, more precisely, "mitochondrial," as their density is particularly high on the outer surface of the mitochondrial membranes (26). No action has yet been ascribed to the binding of BZ to these sites (Table 1).

Diazepam binds with high affinity to both the $GABA_A$ and the mitochondria-linked recognition sites. The BZ Ro5-4864 (4-chlorodiazepam) (3) and the isoquinoline PK11195 (isoquinoline carboxamide) (25–27) are the most potent ligands for the mitochondrial BZ binding sites, although they can also bind to a special class of $GABA_A$ ($GABA_{A3}$) receptor (28) (see Table 1). In contrast, flumazenil, clonazepam, and the beta-carbolines are the most selective ligands for the $GABA_{A1}$ and $GABA_{A2}$ receptor-linked BZ recognition sites: they are virtually unable to bind to the BZ recognition sites located on the mitochondrial membranes (3, 5, 25). The suggestion has been made that the mitochondrial binding sites are associated with an anion channel called "porin," present on the outer layer of mitochondrial membranes (25).

Several lines of independent investigation have suggested that the $GABA_A$ and mitochondrial BZ binding sites might function as receptors for the action of physiologically relevant endogenous ligands. Hence, the search for ligands that bind to both sites, or to either, became a research trend in several laboratories.

Various endogenous constituents isolated from brain and other tissues have been reported to displace specific ligands bound to the $GABA_A$ receptor- or the mitochondria-linked recognition sites for BZ. These compounds include purines (29, 30), nicotinamide (31), beta-carbolines (7, 32), various peptides (22, 33–36), porphyrins (37), and the benzodiazepines themselves (38). However, only a few of these substances appear to possess the attributes essential to being ranked as physiologically relevant ligands. The potency of purines and nicotinamide in displacing BZ from specific recognition sites is very low (K_i in the mmol range). In contrast, the affinity of beta-carboline-3-carboxylate esters for $GABA_A$ receptor-linked BZ sites is quite high (K_i in the nmol range). Traces of such compounds have been detected in brain homogenates (32), but it is unclear whether they are present in physiologically meaningful concentrations (7). Though traces of benzodiazepines have also been found in mammalian brain, they may be of exogenous origin, as potatoes and other agricultural products contain BZ as well (W. Haefely, personal communication). Porphyrins are ligands for mitochondria-linked BZ recognition sites, but the physiological role of these sites is unclear (25).

Among the endogenous BZ ligands, a 10 kd peptide (termed DBI for diazepam binding inhibitor) exhibits a number of characteristics indicative of its being a putative precursor for a family of physiological allosteric modulators of $GABA_A$ receptors, acting at the BZ recognition sites (39, 40). DBI, with 86 amino acid residues, has two processing products, one of which (DBI 33-50) is an 18-amino-acid segment termed ODN, for "octadecaneuropeptide" (41). Binding studies in primary cultures of neonatal rat brain neurons suggest that the portion of ODN responsible for displacement of ^{3}H-BC (41) or ^{3}H-flumazenil is the carboxy terminal region (Table 2). The human (DBI 1-106), rat (DBI 1-86), and cow (DBI 1-86) DBI displace specifically the BZ and BC bound to the $GABA_A$ receptor with a K_i of approximately 5 μM (39, 42, 43). This is probably not the only biological "message" encoded in the DBI molecule. In fact, DBI also inhibits the binding of Ro5-4864 and PK11195 to the BZ sites on mitochondria of astrocytes (Table 2).

Patch clamping in the whole cell mode of the mouse spinal cord neurons in primary culture revealed that rat DBI acts as a negative allosteric modulator of the GABA-operated Cl^- channel opening (44). Thus, like BC, rat DBI also reduces the probability of GABA having an action on the channel (44). Immunocytochemical studies show that DBI-like immunoreactivity is unevenly distributed in the brain, with a region-specific concentration gradient similar to that of the GABA receptor complex (44). Furthermore, DBI has been shown immunocytochemically to colocalize with glutamic acid decarboxylase in primary cultures of rat neurons (8, 45) and by subcellular fractionation studies to be concentrated in synaptic vesicles (46). Though DBI

Table 2 Binding properties of rat DBI and rat DBI fragments

	Peptides		^{3}H-flumazenil binding to cerebellar granule cells	^{3}H-PK11195 binding to astrocyte mitochondria
			K_i (μM)	
DBI (1–86)		SQADFDKAAEEVKRLKTQPTDEEMLFIYSHF KQATVGDVNTDRPGLLDLKGKAKWDSWNKLK GTSKENAMKTYVEKVEELKKKYGI	5	5
DBI (17–50)		TQPTDEEMLFIYSHFKQATVGDVNTDRPGLLDLK	>100	10
DBI (17–41)		TQPTDEEMLFIYSHFKQATVGDVNT	>100	>100
DBI (33–50)	ODN	QATVGDVNTDRPGLLDLK	5	>100
DBI (33–42)		QATVGDVNTD	>100	>100
DBI (42–50)		DRPGLLDLK	30	50
DBI (42–50)-NH_2		DRPGLLDLK-NH_2	>100	>100

The assay of ^{3}H-flumazenil binding was carried out in intact newborn rat cerebellar granule cells in primary cultures (41) using 2 nM ^{3}H-flumazenil. The assay of ^{3}H-PK11195 binding was carried out on crude mitochondria obtained from newborn rat cerebellar astrocytes maintained in culture (52). ^{3}H-PK11195 was 5 nM. Amino acid sequence reported in the one letter notation.

is present in both neurons and glia, it can be released by depolarization in a TTX- and Ca^{2+}-dependent manner only from neurons (46). Since the amino terminus of DBI (extracted from rat or human brain) appears to be blocked, a partial amino acid sequence of rat (39–41) and human (42, 43) DBI had to be obtained by sequencing its tryptic fragments or CNBr cleavage products. From these known sequences, complementary oligonucleotide probes were synthesized (47, 48) and used to hybridize brain cDNA libraries. The positive clones were then analyzed to obtain the following amino acid sequences of rat and human DBI (47, 48).

HUMAN: WGDLWLLPPASANPGTGTEAEFEKAAEEVRHLKTKPSDEEMLFIYGHYK-
QATVGDINTERPGMLDFTGKAKWDAWNELKGTSKEDAMKAYINKVEELKKKYGI

RAT: SQADFDKAAEEVKRLKTQPTDEEMLFIYSHFK-
QATVGDVNTDRPGLLDLKGKAKWDSWNKLKGTSKENAMKTYVEKVEELKKKYGI

The two DBIs have a high degree of sequence homology, suggesting that they are encoded by genes that are phylogenetically quite closely related. Similar considerations apply to bovine DBI (49).

Characterization of human and rat DBI genes by Southern blot analyses suggests that DBI is encoded by multiple gene loci (47, 48). In both rat and human brain, at least five independent bands were hybridized with cDNA probes complementary to DBI, using genomic DNA digested with different restriction endonucleases. Although it appears that some of these bands may represent pseudogenes, it is quite probable that DBI-related polypeptides can be transcribed by more than one gene that lights up with the cDNA probe for DBI. Thus, DBI may be one of the precursors for a family of as yet undiscovered peptides that, coexisting with GABA, allosterically modulate $GABA_A$ receptors by specifically binding to the BZ/BC recognition sites. In addition, DBI or a product from one of the multiple genes hybridized by the cDNA probe for DBI could be a precursor for ligands acting on the other types (i.e., mitochondrial) of BZ recognition sites listed in Table 1.

DBI-like immunoreactivity is found in neurons, selective populations of glial cells (astroglia and cerebellar Bergmann cells), and some peripheral tissues (45–48). Thus, the discovery of multiple genes encoding DBI raises the possibility of cell-specific transcriptional mechanisms. However, it appears that some of the DBI genes lack introns and therefore should be considered pseudogenes transcribed from mRNA by reverse transcriptase. Other DBI sequences that may contain introns may lack promoter and enhancer regions for their transcription. If only one DBI gene is being transcribed for a multiplicity of functionally different BZ recognition sites, then

a cell-specific processing mechanism must be invoked. To sort out this question and to obtain more information on the peptide's functional role, we began to examine DBI processing in neurons and other cells of rats under different experimental conditions.

The presence in the DBI structure of amino acid signals for DBI's tryptic cleavage (see above) is compatible with the view of precise, cell-specific differences in DBI processing. As already mentioned, tryptic digestion of rat DBI yields an octadecaneuropeptide (DBI 33-50) called ODN (see Figure 1, Table 2) that shares some of DBI's biological properties (41, 50, 51). Using polyclonal antibodies raised against ODN and DBI, we demonstrated that DBI coexists in rat brain or in primary culture of neonatal rat cortical neurons with two major processing products that immunoreact with ODN antibodies (52). Double immunofluorescence staining of these neurons with glutamic acid decarboxylase (GAD) and ODN antibodies indicates that ODN and ODN-like peptides are colocalized with GAD in at least 50% of the cultured cortical neurons (52). Moreover, GABA and ODN-like peptides are coreleased following veratridine-induced depolarization (52).

In astroglial cells, the mitochondrial membranes are densely packed with BZ recognition sites. DBI and DBI mRNA are expressed, but DBI does not appear to be compartmentalized in subcellular particles. Furthermore, neither ODN nor ODN-like peptides can be detected as a DBI processing product, and DBI is not released by depolarization (52). We therefore suggest that DBI

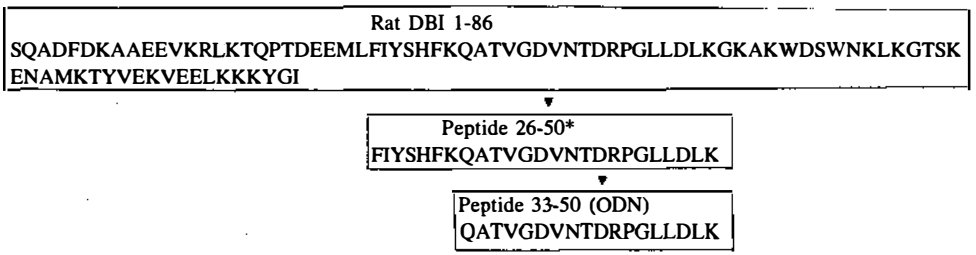

Figure 1 Amino acid sequence of two endogenous allosteric neuromodulators of the $GABA_A$ receptor and their precursor polypeptide (DBI)

*Peptide 26-50 has been purified from rat brain by Dr. E. Slobodyansky in our laboratory (personal communi tion).

may undergo different processing and have different functions in neurons and glial cells. Neuronal DBI is compartmentalized in synaptic vesicles, processed to ODN-like and other peptides located in synaptic vesicles, and released extracellularly by exocytosis following neuronal depolarization. Glial DBI is not processed to ODN-
extracellularly, and could be acting on the mitochondrial-linked BZ recognition sites involved in the regulation of anion transport across the mitochondrial membranes.

To elucidate the possible role of DBI in glial elements and its relationship to BZ recognition sites, we used a culture of neonatal rat cerebral cortical astrocytes. We have found that in these cultures BZ binding sites recognize with high affinity (kd ~ 5 nM) and large capacity (B_{max} 20 pmol/mg prot) both the isoquinoline derivative PK11195 and the benzodiazepine derivative Ro5-4864 (Table 1). Highly localized in the mitochondrial fraction, the binding of ^{3}H-PK11195 to the astrocytes is neither regulated by the addition of GABA nor displaced by flumazenil. The molecular weight M_r of the recognition-site polypeptide subunit photolabeled with ^{3}H-PK14105 is 17 kd (53). Interestingly, this MW is identical to that of the corresponding subunit in mitochondria of adrenal cortex, liver, and other peripheral organs; however, protoporphyrins, agents that displace ^{3}H-PK11195 from adrenal or liver mitochondrial membranes (K_i in the nM range) (37) fail, up to 10 μM, to displace ^{3}H-PK11195 from the astroglial mitochondria. The recognition site for ^{3}H-PK11195 or Ro5-4864 was isolated and purified to apparent homogeneity by photolabeling with ^{3}H-PK 14105 followed by ion exchange chromatography and HPLC and found to be a 17 kd polypeptide. This peptide may represent one of the elementary subunits of a receptor complex whose M_r has been estimated to be around 50–60 kd (54). DBI competitively inhibited the binding of PK11195 and Ro5-4864 to crude mitochondrial preparations of astrocytes with a K_i of approximately 5 μM. The peptide is not synthesized by mitochondrial DNA, which may explain the different quantities of it in various cell types (53).

To establish the structural and chemicophysical properties of DBI relevant to the PK11195 binding sites, and how these properties differ from those required for binding to the $GABA_A$ receptor complex, we tested several synthetic DBI peptide fragments for their ability to displace the inert ligand PK11195 from crude mitochondrial astrocytes and flumazenil from $GABA_A$ receptors in cultured cortical neurons of neonatal rats. ODN (DBI 33-50) displaces ^{3}H-flumazenil from the $GABA_A$ receptor with an affinity of approximately 5 μM, but fails to displace ^{3}H-PK11195 from the astrocytic mitochondria (Table 2). In contrast, the DBI 17-50 fragment is capable of displacing ^{3}H-PK11195 (K_i~10μM) but not ^{3}H-flumazenil (up to 100 μM)

(Table 2). DBI 42–50 (containing the nine carboxy terminus amino acid residues of DBI 21-50 or 33-50) maintains the ability to displace the binding of both ligands, though with lower affinity. Interestingly, the amide of DBI 42-50 is devoid of activity. Moreover, DBI 17-30 and 33-42 (see Table 2) fail to inhibit the binding of either ligand. We conclude from these observations that the carboxy terminal region of ODN (DBI 33-50) as contained in DBI 42-50 represents the essential unit for the "message" at the BZ binding sites coupled to the $GABA_A$ receptor and to the mitochondrial astrocytes. The amino terminal region of ODN (DBI 33-42), which in computer-assisted analyses of the DBI hydropathy spectra appears to be hydrophilic, and the amino terminal region of DBI 17-50 (DBI 17-30), which appears hydrophobic and capable of assuming an alpha helical configuration, may be involved in promoting the proper orientation (address) of the "message" region (the carboxy terminus) for recognition of the BZ binding sites.

Binding and pharmacological experiments have shown that ODN acts on a site for the negative allosteric control, of the $GABA_A$ receptor (41, 50, 51). This suggests that ODN and other ODN-like peptides derived from DBI might participate, with physiological significance, in decreasing the probability that quanta of GABA released in the synaptic cleft will open specific Cl^- channels on the postsynaptic cell membranes. The structure of ODN-like peptides in rat brain neurons has been investigated. One of these peptides has an HPLC retention time identical to that of the synthetic ODN. The major peptide fragments that immunoreact with ODN antibodies, however, have a different retention time on reverse-phase HPLC. We have recently purified one of these peptides with immunoaffinity column chromatography. Analysis of the amino acid sequences indicates that it is a product of DBI processing (DBI 26-50), an amino terminus elongated form of ODN. The analysis also revealed a chain of seven amino acids extending at ODN's amino-terminal portion (see Figure 1). Preliminary pharmacological studies indicate that the potency of DBI 26-50 may be higher than that of ODN itself. Perhaps the chain of seven amino acids (see Figure 1) confers on ODN a hydrophobic portion with an alpha helical configuration. This alpha helix may facilitate the interaction of the ODN carboxy terminal region with the amphiphilic biological interface of the neuronal membrane receptors.

We used two strategies to acquire information on the role of DBI/ODN-like peptides in the control of brain function: (*a*) studies of the dynamic state of DBI in neurons and other cells under different experimental conditions; and (*b*) measurement of DBI/ODN-like peptides in cerebrospinal fluid (CSF) of normal individuals and patients with various neuropsychiatric disorders.

For the first strategy, a cDNA probe specific for rat brain DBI (48) enabled us to assess either the content of the DBI mRNA by RNA Northern blot analyses or the location of DBI mRNA by in situ hybridization techniques.

Antibodies raised against DBI (45) were used to measure changes of DBI content, which could then be correlated with the changes in mRNA content. Finally, antibodies raised against ODN (55) helped to assay the content of ODN-like immunoreactivity in tissues. Correlation of these values could be expected to reveal whether a change in the content of DBI and of its mRNA is associated with an alteration of DBI synthesis or with processing to ODN-like immunoreactive peptides. In one experimental approach, rats were treated repeatedly with diazepam (10 days, 3 times a day by oral gavage with 10 to 20 mg doses). This treatment yields tolerance to the acute sedative and ataxic effects of benzodiazepines, induces physical dependence, and evokes a withdrawal reaction when diazepam is discontinued abruptly. The molecular mechanisms of BZ tolerance and withdrawal are unclear. However, the two phenomena are not due to change in the number of BZ recognition sites. Instead, they may result from functional GABA-receptor down regulation (56, 57).

We found that during BZ tolerance the content of DBI, DBI mRNA, and ODN-like immunoreactive peptides increased in cerebellum and cortex but failed to change in the hippocampus and striatum (55). Furthermore, in cerebellum, the increase of ODN-like peptides was five times larger than that of DBI or DBI mRNA, suggesting that tolerance to BZ is associated with an increase in the turnover rate of DBI. This increase may in turn be either responsible for, or due to the desensitization of GABA receptors that occurs after protracted benzodiazepine administration. (It is important to note that neither a single administration nor several other dosage regimens of BZ, repeated for several days, elicited BZ tolerance or any change in the content or dynamic state of DBI.)

In experiments conducted along the lines of the second research strategy, we compared the CSF content of DBI in normal human subjects and in patients suffering from depression, schizophrenia, and dementia of Alzheimer's type, all of which may be associated with functional alterations of the $GABA_A$ receptor (58). As shown in Table 3, the CSF of patients with severe forms of major depression contained significantly higher concentrations of DBI than that of age- and sex-matched normal volunteers. In contrast, the CSF of schizophrenics and patients with dementia of Alzheimer's type showed no differences in DBI concentrations compared to controls.

There is both direct and indirect evidence for decreased GABAergic function in various forms of endogenous depression (59). Consistent with this hypothesis, both direct and indirect-acting GABA-mimetic drugs have been found to be effective antidepressants (59). Thus, an increase in DBI immunoreactivity in the CSF of depressed patients could reflect an impairment of central GABAergic neurotransmission. Studies of DBI immunoreactivity in the spinal fluid of individuals with manic-depressive disorder, as well as of

Table 3 CSF levels of DBI immunoreactivity in depressed patients and control subjects

Subject	Age	Sex	DBI (pmol/ml CSF)
Normal	36 ± 2.7	6 Female 4 Male	1.1 ± 0.09
Depressed	36 ± 2.5	6 Female 4 Male	1.4 ± 0.13*

*$P < 0.03$.

Patients were diagnosed according to the DSM III criteria and were free of all medications for at least two weeks prior to study.
For details on diagnosis and methods see Barbaccia et al (58).

those treated with antidepressant medication, may establish whether the elevated concentration of DBI is a state or a trait in depression.

The rather obscure relationship between changes in the content of DBI in spinal fluid and the dynamic state of DBI in the brain is under further investigation.

ALLOSTERIC MODULATION OF EXCITATORY AMINO ACID RECEPTORS

Role of Endogenous Ligands for Phencyclidine (PCP) Binding Sites

The discovery of benzodiazepine-GABA interactions, first described in 1975 by Haefely et al (60) and Costa et al (61), led to the development of two new basic concepts in synaptic communication: polytypic signaling at synapses and allosteric modulation of transmitter receptors (4, 8). In addition, it stimulated research into the role of GABA in conflict behavior (40). Furthermore, the discovery yielded knowledge about the pharmacology of GABA-initiated signal transduction, leading to the hypothesis that high-affinity receptor recognition sites for psychotropic drugs play a role in brain physiology (8).

Phencyclidine binds with high affinity to one such recognition site, presently associated with one type of synaptic receptor complex for dicarboxylic excitatory amino acids (62–65). The drug phencyclidine, or PCP (1-[phenylcyclohexyl] piperidine), which is currently widely abused, was first employed as an anesthetic; clinicians discontinued the drug after a few years because of its dissociative effects. In abusers of PCP these effects manifest themselves in loss of memory and a schizophrenia-like syndrome (66).

A number of reports (62–64, 67) have described the stereospecific and

saturable binding of PCP or PCP derivatives to membranes prepared from rat brain and from primary cultures of rat cerebellar granule cells. Interestingly, the binding sites are relatively abundant in the hippocampus and cortex (67), suggesting that a functional relationship with the psychotomimetic effects of PCP cannot be excluded. Behavioral (68, 69) and electrophysiological (70–74) tests have demonstrated the same rank order for psychotomimetic and ^{3}H-PCP-displacing potency for various PCP congeners. In contrast, a number of transmitters and peptides believed to be neuromodulators failed to displace PCP at these binding sites (62–67), implying that the PCP is occupying the site where an as yet unknown neuromodulator binds.

Some investigators have suggested that PCP's psychotomimetic effects result from its interaction with the sigma recognition sites for a putative sigma opioid ligand (67); however, recent experiments show that metaphit, an acylating ligand for the ^{3}H-PCP recognition site, can block the behavioral effects of PCP but not those of cyclazocine or SKF 10,047, two ligands selective for the sigma binding sites of opioids (75). Thus, it is doubtful that the ^{3}H-PCP and sigma opioid recognition sites are identical.

Evidence is now accumulating for a functional and perhaps anatomical connection between the ^{3}H-TCP (a thienyl derivative of PCP) binding sites and the receptors for excitatory dicarboxylic amino acids. The regional distributions of the ^{3}H-TCP and ^{3}H-glutamate recognition sites displaced by N-methyl-D-aspartate (NMDA) are similar (65). Biochemical and electrophysiological studies in primary cultures of rat cerebellar granule cells, in cortical neurons, and in whole animals have shown that PCP and its congeners noncompetitively inhibit signal transduction at the Mg^{2+}-sensitive NMDA-preferring glutamate receptors (62, 70–72, 76–78). These receptors have been divided into two classes (78): GP_1, so named because its occupation by the appropriate ligand results in the activation of phosphoinositide (PI) breakdown; and GC_1, occupation of which causes the opening of specific cationic channels. PCP blocks both these classes of receptors but it does not have a significant effect on two other classes of "glutamatergic" receptors that are not inhibited by Mg^{2+} (62): GP_2, which is preferentially activated by quisqualate and coupled to PI turnover via a G protein; and GC_2, which is preferentially activated by kainate and operates a cationic channel with properties different from those of the channel operated by the GC_1 receptor subtype.

All of these data taken together suggest that PCP when bound to recognition sites associated with the GP_1 and GC_1 receptors may be a noncompetitive antagonist of glutamate-elicited signal transduction (62).

Glutamatergic transmission at GC_1 receptors, like that of GABA at $GABA_A$ receptors, can be influenced by a positive allosteric modulator. Johnson & Ascher (79) have reported that glycine, at concentrations that per se do not affect the synaptic current elicited by the opening of NMDA- and glutamate-operated cationic channels, can greatly potentiate the effect of a

threshold concentration of NMDA when bound to its strychnine-insensitive receptor subunit. Bertolino et al have extended the significance of this finding by showing its importance for activity at the single-channel level (80). Moreover, glycine, like PCP, modulates only the GC_1 and GP_1 glutamate receptors, having no effect on GC_2 or GP_2 receptors (79, 80). Since modulation by PCP of the GC_1 cationic channels appears to be voltage-sensitive, while that by glycine is not, it can be inferred that PCP and glycine interact with two different sites associated with the channel. Thus, the $GABA_a/Cl^-$ channel and the glutamate (GC_1)/cationic channel receptor complexes appear to be very similar in their positive allosteric modulation but different in their negative modulation. Glycine acts in the same way as the anxiolytic benzodiazepines: both increase the probability that the transmitters will open their respective ionic channels. In contrast, PCP acts differently from the beta-carbolines: instead of decreasing the probability of channel opening, PCP decreases the amount of time the channel is open. This difference might explain why the $GABA_A$ receptor becomes desensitized after prolonged exposure to GABA while desensitization of the GC_1 receptor to glutamate has not been reported to occur.

Glycine is a natural compound and may thus serve as a putative "endogenous" ligand. PCP and congeners, however, are synthetic molecules. Attempts have been made to ascertain whether—as is the case for other drug-specific receptors—an endogenous ligand for the PCP low- or high-affinity binding site exists that induces biochemical and/or behavioral effects similar to those of the synthetic PCP. Quirion et al (81) did find a substance(s) in acidic extracts of porcine brains that selectively inhibited ^{3}H-PCP-specific binding to membranes prepared from rat brain. The biological activity of this substance, measured by its ability to inhibit ^{3}H-PCP binding, was greatly reduced by pronase, trypsin, and carboxypeptidase A, and unaffected by alpha-chymotrypsin. The apparent molecular weight of this peptide was judged to be approximately 3 kd, based on molecular sorting with gel filtration chromatography. The peptide is highly concentrated in porcine hippocampus and frontal cortex; 5–50 times less was found in cerebellum and corpus striatum and brain stem. This putative endogenous ligand is selective for the ^{3}H-PCP binding site—it does not inhibit the binding of ligands to mu, delta, and kappa opioid recognition sites or to benzodiazepine or neurotensin high-affinity recognition sites. Of great interest is the observation that this peptide can be chromatographically differentiated from another peptide in porcine brain extract that selectively inhibits binding of ^{3}H-SKF 10,047, a selective ligand for the sigma opioid recognition sites (82). Thus, it would seem that PCP binds to two recognition sites: one with lower affinity, sharing qualities with the sigma opioid receptor; and another with higher affinity, presumably associated with GC_1 and GP_1 glutamate receptors. Though the

amino acid structure of the two peptides is not yet known, Contreras et al (82) suggest that the two putative endogenous ligands may be structurally related through derivation from the same precursor, which is processed differently in various types of neurons.

The putative endogenous ligand for ^{3}H-PCP binding sites also elicits PCP-like responses in some electrophysiological and behavioral tests, but whether it actually modulates glutamatergic neurotransmission is unknown. Indirect evidence on the regional distribution of the ^{3}H-PCP and ^{3}H-glutamate (NMDA-displaceable) bindings and on the location of the peptide itself indicate a possible functional interplay among these elements. At this time we know of no direct experiment that has examined whether the ^{3}H-PCP-binding peptide interacts with the GP_1 and/or GC_1 recognition sites for L-glutamate. Needless to say, if this were the case, one would have a potentially powerful pharmacological tool for probing the phenomenon of cell memory in experimental models such as long-term posttetanic potentiation in the hippocampus, where glutamatergic transmission plays a key role.

DO ENDOGENOUS MODULATORS FOR PRE- AND POSTSYNAPTIC SEROTONERGIC RECEPTORS EXIST?

Putative Endogenous Ligands for the ^{3}H-Imipramine and ^{3}H-Ketanserin Recognition Sites

High-affinity recognition sites for imipramine, a typical tricyclic antidepressant, were first described by Raisman et al (83). Labeling with ^{3}H-imipramine revealed binding sites in membrane preparations from the brains of rat and several other species as well as in peripheral tissues and blood platelets (84–88). The binding sites in brain and platelets appeared to be almost identically displaceable by a number of tricyclic antidepressant drugs as well as by serotonin-uptake inhibitors and serotonin (5-hydroxytryptamine, or 5HT) itself (88–90).

Several lines of experimental evidence suggest an anatomical and functional association between the 5HT uptake sites and ^{3}H-imipramine binding sites: (*a*) highly significant positively correlated K_i values for inhibition of 5HT uptake and ^{3}H-imipramine binding (84–90); (*b*) a regional distribution of these sites paralleling 5HT innervation (91); and (*c*) dramatic decrease of high-affinity ^{3}H-imipramine sites caused by selective lesioning of 5HT axon terminals (91–93). Still, the sites for 5HT and for imipramine are probably not identical (94, 95).

The B_{max} of high-affinity ^{3}H-imipramine recognition sites in several brain regions can be decreased by repeated daily treatment (10–20 days) with imipramine or desipramine (95–99)—interestingly, one sees a concomitant

increase in the V_{max} of ^{3}H-5HT uptake in minces of hippocampal tissue. A similar dissociation between 5HT uptake and ^{3}H-imipramine binding has been found in human platelets (100–102). These and other observations, such as the decreased B_{max} of the high-affinity ^{3}H-imipramine recognition sites and/or decrease in 5HT uptake found in platelets from untreated or drug-free severely depressed patients (100, 101, 103–107), led to the hypothesis that there is an endogenous substance(s) with a high affinity for the ^{3}H-imipramine binding site, the content of which is decreased or increased following pharmacological manipulation or during psychopathological states. Several laboratories have attempted to extract and purify such a ligand, or endacoid (from the Greek *endos*:inside, and *akos*:drug), from two main sources: human biological fluids (mainly plasma) and rat brain tissue.

Extraction and partial purification of substances from human plasma have yielded several candidate ligands. Angel & Paul (108) have described a heat-stable factor(s) (MW$\leq$ 10,000 daltons) that is sensitive to protease digestion and selectively inhibits ^{3}H-5HT uptake from rat synaptosomes in a reversible and apparently noncompetitive fashion. Unfortunately, their paper contained no data on the inhibition of ^{3}H-imipramine binding by the same factor(s). Brusov et al (109) have also extracted low-molecular-weight substances capable of inhibiting both ^{3}H-imipramine binding and ^{3}H-5HT uptake from human plasma. The biological activity of this factor (s) depended to a degree on the presence of intact peptide bonds. Pretreatment of the extract with carboxy-peptidase B or leucine-aminopeptidase partially prevented the inhibition of binding but had a much weaker effect on uptake. Upon fractionation of this extract on a Biogel P_2 column, at least four different peaks of inhibitory activity of both binding and uptake were resolved. Since no further characterization of these four different peaks has been reported, we do not know if they are all specific for the inhibition of ^{3}H-imipramine binding and 5HT uptake, all peptidergic, or whether they are structurally related or distinct from each other. More recently (110), there has been a report of purification from human plasma of a glycoprotein (weighing approximately 45,000 daltons) that displays several physicochemical properties very similar to those of the alpha 1-acid-glycoprotein normally present in human plasma; the two proteins copurified under various chromatographic conditions. This glycoprotein inhibits with an IC_{50} of approximately 6μM the binding of ^{3}H-imipramine to human platelets but increases (EC_{50} approximately 7 μM) the ^{3}H-5HT uptake into human platelets. However, platelet-free plasma may contain another substance capable of inhibiting ^{3}H-5HT uptake. Abraham and co-workers (110) detected such inhibitory activity, but it has not been further characterized. Moreover, the authors did not report any evidence on the ability of this glycoprotein to inhibit ^{3}H-imipramine binding and ^{3}H-5HT uptake in brain tissue.

Rat brain constituents with an apparent low molecular weight appear to be the candidate endacoids for the imipramine binding site. The physicochemical characterization and purification to homogeneity of a substance in rat brain has been obtained, but the identification of its molecular structure has presented some difficulties. Barbaccia et al (111, 112) and Rehavi et al (113), each group using a different procedure, reported that a low-molecular-weight (< 1000 daltons) substance extracted and partially purified from whole rat brain could selectively inhibit imipramine binding in membranes and 5HT uptake in synaptosomes. These preparations contained no endogenous serotonin. Moreover, the substance's inhibitory activity was not abolished by pretreatment with various proteases. Though caution is warranted in interpreting this kind of data, several lines of evidence (111, 112) support the view that the inhibitory activity is not due to nonspecific factors such as hyperosmolarity, as suggested by Lee et al (114). In fact, inhibition of binding and uptake was abolished when the extract was treated with strong acids (HCl, $HClO_4$); furthermore, this putative endacoid was shown to be unevenly distributed in various regions of the rat brain (112). Though it has been previously reported that the extract could not be satisfactorily chromatographed on a reverse-phase HPLC column, a considerable enrichment in biological activity was seen when the extract was electrophoresed on agarose under high voltage (1,100 volts) and subsequently applied to a cation-exchange HPLC column (111). Thin-layer chromatography on silica gel of the active peak after this mode of purification yielded three spots detectable with iodine. In view of its electrophoretic behavior and its insolubility in propanol, acetonitrile, ether, and chloroform, this putative endacoid is most likely polar.

The concentration of the extract that is needed to inhibit paroxetine binding at 37°C is 10 times higher than that needed for the inhibition of imipramine binding at 0°C. Although the reason for this discrepancy is not known, this "temperature effect" is quite similar to what happens with other tricyclic antidepressants, as compared to nontricyclic inhibitors of 5HT uptake (115). In any case, the fact that the extract is at least 10 times more potent in inhibiting 5HT reuptake than norepinephrine reuptake suggests that this biological activity is specific for the imipramine/5HT reuptake complex. We are analyzing this purified extract with mass fragmentographic techniques to gain insights into the molecular nature of this putative endogenous ligand. (Thus far a number of known substances have been ruled out as candidates to be the putative endacoid, based on their apparent inability to inhibit imipramine binding and 5HT uptake [96, 116]).

The identification and characterization of the putative endogenous ligand for the imipramine recognition site could have great meaning for research in biological psychiatry. Assuming that the ligand plays a physiological role in

controlling the gating of the 5HT reuptake mechanism, monitoring of its fluctuations following pharmacological interventions or during psychopathological states could shed new light on the dynamics of serotonergic transmission, which is believed to play a key role in the response to treatment with antidepressants and possibly in the pathogenesis of mood disorders (117).

One hypothesis now being tested is that serotonergic neurotransmission can be modulated not only presynaptically (i.e. by the putative endogenous ligand for the imipramine binding site) but also postsynaptically. This idea gained popularity after the coexistence in the same neurons of serotonin with substance P and/or TRH (thyrotropin releasing hormone) was described (118, 119), the assumption being that a classical transmitter and a peptide coexisting in the same neurons could be coreleased, with one acting as a postsynaptic modulator of the other. However, no experimental design thus far has unequivocally succeeded in demonstrating such a modulation of the 5HT receptor function by either substance P or TRH.

One of the more intriguing aspects of 5HT receptors is the function and regulation of the so-called "$5HT_2$" recognition sites, which are labeled by ^{3}H-LSD, ^{3}H-spiperone (120, 121), and the more selective ^{3}H-ketanserin (122). Despite their name, these sites are not up-regulated upon selective chemical or surgical denervation of the serotonergic afferent fibers (123, 124), as is the case for other serotonergic receptors (125). Furthermore, the transducer mechanism (phosphoinositide hydrolysis through the activation of phosopholipase C), which appears to be coupled to $5HT_2$ recognition sites in rat brain cortical tissue, does not seem to be sensitized by lesioning 5HT axon terminals (126). Nevertheless, ketanserin and other selective $5HT_2$ ligands do inhibit phosphoinositide hydrolysis (126) and also some behavioral effects (127) elicited by 5HT, suggesting that the $5HT_2$ binding site is really part of the supramolecular organization of the postsynaptic 5HT receptor. The density of $5HT_2$ sites is decreased following "in vivo" treatment with many known antidepressants (123, 128–133) (conversely, repeated electroconvulsive shock increases their density [134]), with a time course that correlates well with the lag time of the appearance of the therapeutic effect. This suggests a role for these sites in the pharmacological action of the drugs. It is also worth noting that "in vivo" treatment with several 5HT antagonists (ketanserin, ritanserin, setoperone, mianserin, LSD, methergoline) reduces the number of binding sites specifically labeled by ^{3}H-ketanserin in the frontal cortex (130, 133, 135). It is not yet clear why chronic exposure to an antagonist should elicit down regulation of the receptor—perhaps these drugs are "antagonists" as far as 5HT-elicited effects are concerned, but "agonists" when bound to the $5HT_2$ sites. One could assume that (*a*) 5HT is not the endogenous ligand for the sites labeled by ketanserin, and that (*b*) another

endogenous substance exists that physiologically binds to these recognition sites. If these assumptions turn out to be true, then this putative novel substance, by binding to the ketanserin-labeled site, would function either as an allosteric modulator or as a cotransmitter of the serotonergic postsynaptic receptor, depending on whether it turns out to have any intrinsic activity on its own.

We have extracted and purified from bovine and rat brain a peptide that selectively inhibits ^{3}H-ketanserin binding (111). This peptide, the partial amino acid sequence of which is now being analyzed, has been purified through gel filtration, cation-exchange, chromatography, and HPLC. Its apparent molecular weight is between 15 and 16 kd. It inhibits the specific binding of ketanserin much more strongly than that of either mainserin or imipramine and thus can be described as more, if not completely, selective for $5HT_2$ recognition sites than for $5HT_1$. Still, we do not know whether the peptide has any intrinsic activity that would give it a profile similar to that of ketanserin or serotonin.

CONCLUSIONS

The presence in GABA-operated Cl^- channel of an allosteric center for the positive and negative modulation of the primary transmitter recognition site has prompted the suggestion that the transaction of GABA-mediated synaptic transmission involves a polytypic chemical signaling. This possibility is corroborated by the coexistence in the same axon terminals of GABA and DBI processing products. Since these peptides displace radioactive BZ and/or BC from their high affinity binding sites located in the allosteric modulatory center of GABA receptors, and since depolarization coreleases GABA and DBI fragments, these peptides are considered the endogenous allosteric modulators of GABAergic transmission. Indeed, flumazenil, the specific antagonist of BZ and/or BC, modifies the operation of GABA-gated chloride channels following transynaptic activation, but not its operation after direct application of GABA. Taken together, these data uphold the importance of polytypic signaling at GABAergic synapses. A similar multiple signaling applies to glutamatergic receptors and perhaps to other transmitter receptors (5HT, catecholamines, acetylcholine, etc.). Hence, this research trend has promoted the pharmacology of the modulation of synaptic transmission. Its basic tenet is that centrally acting drugs should be directed to the natural mechanisms of allosteric modulation rather than to the mechanism of isosteric inhibition of primary transmitter transduction.

Drugs acting as allosteric modulators (positive or negative) have a number of advantages over those acting as isosteric competitive inhibitors. They fail to trigger compensatory mechanisms of synaptic plasticity, such as the receptor supersensitivity or subsensitivity caused by the isosteric antagonists and

agonists, respectively. These modifications may lead to iatrogenic dysfunctions or malfunctions of transmitter receptors, as is exemplified by the tardive dyskinesia that follows the protracted use of isosteric competitive inhibitors of dopamine receptors. It is conceivable that drugs acting as "allosteric modulators" of GABA receptors through an increase or decrease of the GABA-mediated transduction mechanism would not alter the rhythm of physiologically evoked signals, or cause permanent alteration of primary transmitter receptors. Since it is now known that endogenous allosteric modulators are physiologically operative in synaptic transmission, it is possible to devise a new generation of drugs that would modify their synthesis, release, storage and receptorial action.

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