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REVIEW ARTICLE

Biochemical dissection of the γ-aminobutyrate synapse

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Introduction

γ-Aminobutyric acid (4-aminobutanoic acid; GABA) is the major inhibitory neurotransmitter in the brain. It was originally identified as the principal agent in brain extracts capable of inhibiting crayfish stretch-receptor neurons (Florey, 1954; Bazemore *et al.*, 1957), an effect mediated by an increase in the membrane permeability to Cl^-. With the further demonstration that GABA is released in a calcium-dependent manner from inhibitory nerve fibres in lobster muscle, it became clear that GABA was a prime candidate as an inhibitory transmitter in crustacea (Otsuka *et al.*, 1966). Since that time, a similar role has been established for GABA in the mammalian central nervous system, where it may function at up to 40% of the synapses in brain (Fonnum & Storm-Mathisen, 1978). It is therefore not surprising that neurotransmission at synapses employing GABA has provided a target for a variety of centrally active drugs and toxins (for review see Olsen, 1981). Aberrations of inhibitory transmission have been implicated in a number of neurological disorders, for example epilepsy and Huntington's disease (Tower, 1976; Meldrum, 1978; Lloyd *et al.*, 1977*a*). The molecular actions of two major classes of drugs, the benzodiazepine tranquillizers and the barbiturates, have provided considerable insight into the organization and regulation of the inhibitory synapse at the biochemical level. The purpose of this review is to present current thoughts on the molecular mechanisms by which GABA exerts its inhibitory actions within the nervous system. The structures of several compounds relevant to this article are given in Fig. 1.

Pre-synaptic events: the formation and metabolism of GABA

The organization of 'classical' synapses employing established transmitters such as acetylcholine or catecholamines does not provide a wholly adequate model for comparison with the nerve terminal releasing GABA. It is pertinent therefore to consider briefly the formation and inactivation of GABA in the central nervous system. GABA is widely distributed throughout the brain and is present in substantially higher concentrations (2–4 μmol/g of brain) than many other neurotransmitters (Enna & Snyder, 1976). In this respect it more closely resembles the neuro-excitatory agents glutamate and aspartate. GABA is also present in some peripheral tissues and is particularly high in concentration in reproductive organs (Erdö *et al.*, 1982). The biosynthesis of GABA from glutamate in brain is effected by the cytoplasmic enzyme glutamate decarboxylase (EC 4.1.1.15), which has proved a valuable immunocytochemical marker for GABA neurons (Barber *et al.*, 1978). Significant reduction in the concentration of GABA by inhibition of the decarboxylase markedly increases the susceptibility of animals to convulsive seizures (Wood, 1975). Thus the hyper-polarizing effect of GABA on neurons is crucial to maintain the overall electrical activity of the central nervous system at a sub-convulsive level.

After its removal from the synapse by uptake into neuronal and glial cells, GABA is inactivated by transamination to succinic semialdehyde (Fig. 2). Inhibition of the relevant transaminase (4-aminobutyrate:2-oxoglutarate aminotransferase, EC 2.6.1.19) protects GABA against degradation and may therefore promote neuronal inhibition. As a consequence, numerous inhibitors of the transaminase have received attention as potential anticonvulsant agents (Turner & Whittle, 1980). Succinic semialdehyde is further oxidized to succinate by a specific dehydrogenase (succinic semialdehyde dehydrogenase, EC 1.2.1.16) thereby returning the carbon atoms of GABA to the tricarboxylic acid cycle. The proportion of the total flux through the cycle which goes via the 'GABA-shunt' bypass approaches 10% (Balazs *et al.*, 1970). An alternative route of metabolism for succinic semialdehyde involves reduction to 4-hydroxybutyrate (Turner & Whittle, 1980). Although the reductive pathway to 4-hydroxybutyrate is of lesser importance in quantitative terms, this metabolite has received considerable attention in recent years since it may have a neurophysiological role in its own right (see below and Marcus *et al.*, 1967).

Abbreviations used: GABA, γ-aminobutyric acid; β-CCE, β-carboline 3-carboxylate ethyl ester; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol.

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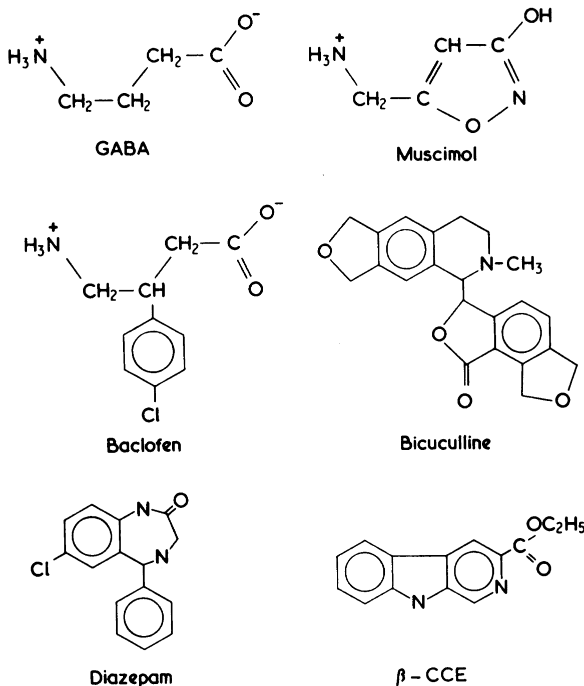

Fig. 1. *Compounds interacting with the receptor complexes for GABA*

Post-synaptic events

The $GABA_A$ receptor is coupled to a chloride ionophore

The best-established response of a neuron to released GABA involves a rapid and transient increase in membrane permeability to Cl^- with resultant hyperpolarization. This change in anion flux is a consequence of the interaction of GABA with a post-synaptic membrane receptor (Enna & Snyder, 1975). The physiological effect may be blocked by bicuculline (Fig. 1), an antagonist of GABA, or by the plant alkaloid picrotoxinin which inhibits the increase in Cl^- conductance mediated by GABA (Takeuchi & Takeuchi, 1969). These bicuculline-sensitive sites for GABA have been the most thoroughly investigated in kinetic and molecular terms and have been designated $GABA_A$ receptors (Table 1; Hill & Bowery, 1981). They are composed not only of the GABA receptor and associated ionophore but may also be coupled to one or more protein components capable of modulating both the availability and affinity of the GABA sites. The potentiation of GABA-mediated inhibition by benzodiazepines, barbiturates and certain other classes of tranquillizers and anticonvulsant drugs is effected through this receptor complex (Olsen,

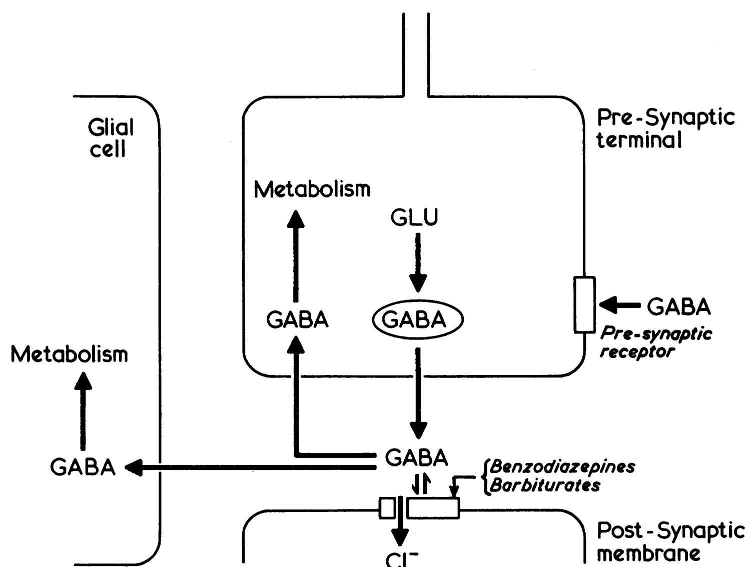

Fig. 2. *Schematic representation of the GABA synapse*

The Ca^{2+}-dependent, evoked release of GABA from the nerve terminal may be regulated through pre-synaptic receptors for GABA (or other neurotransmitters). The post-synaptic response to GABA, an increase in Cl^- permeability resulting in hyper-polarization, can be potentiated by benzodiazepines, anaesthetic barbiturates and certain other drugs (Olsen, 1981), acting through the receptor complex. This response is blocked by bicuculline and picrotoxinin (see the text). Removal of GABA occurs by Na^+-dependent uptake into neuronal and glial cells where the amino acid is inactivated through metabolism to succinate or 4-hydroxybutyrate (see, e.g., Turner & Whittle, 1980).

Table 1. *Sites of drug action at GABA and benzodiazepine receptor complexes*
This list is only illustrative and a more complete listing can be found in Olsen (1981). See the text for further details.

Receptor site	Agonist	Antagonist	Comments
$GABA_A$	GABA, muscimol, 3-aminopropanesulphonate, isoguvacine, THIP	Bicuculline	Order of potency of agonists may vary at pre- and post-synaptic sites. Baclofen is ineffective.
Benzodiazepine	Diazepam (Valium), flunitrazepam, chlordiazepoxide (Librium)	β-Carbolines, e.g. β-carboline 3-carboxylate ethyl ester	Inosine, hypoxanthine, nicotinamide displace [³H]diazepam at mM concentration but physiological relevance unknown.
$GABA_B$	GABA, Baclofen	5-Aminovalerate*	Probable pre-synaptic localization. Muscimol and 3-aminopropanesulphonate have very weak agonist activity. Isoguvacine and THIP are ineffective.
Other	Pentobarbital and other barbiturates, Etazolate, ethanol	Picrotoxinin	Precise site of action not determined. Possible involvement with ionophore component, but see the text.

* 5-Aminovalerate is only a weak competitive antagonist at $GABA_B$ sites (EC_{50} approx. 10^{-4} M; Muhyaddin *et al.*, 1982).

1981). There may be, in addition, a cation recognition site associated with the complex (Squires & Saederup, 1982).

Evidence for the existence of other classes of GABA recognition site, which may not be linked to changes in Cl^- conductance, will be described below. There are also a limited number of examples in which GABA can exert a depolarizing response on nerve fibres (Bowery & Brown, 1974; Brown & Marsh, 1978; Squires & Saederup, 1982).

Kinetics of receptor binding

The first reports demonstrating the specific binding of ³H-labelled GABA to synaptosomal membranes appeared in 1973 (Peck *et al.*, 1973), since when assay conditions have been refined and standardized in many laboratories. An essential requirement is the use of sodium-free buffer solutions to minimize interference from binding to the sodium-dependent transport protein for GABA. Because of the high endogenous levels of GABA, it is also important to ensure that the membrane preparations are thoroughly washed before assay (Napias *et al.*, 1980; Gardner *et al.*, 1981). Under these conditions the sodium-independent binding of GABA to synaptic membranes is consistent with the pharmacological characteristics of post-synaptic receptors. Compounds able to mimic the physiological effects of GABA [e.g. the toxin muscimol (3-hydroxy-5-aminomethylisoxazole), Fig. 1] are able to displace [³H]GABA from binding sites in membrane preparations. Those compounds shown to be more active than GABA in electrophysiological studies are also found to be the most potent inhibitors of binding (Greenlee *et al.*, 1978; Hyttel, 1979; Lloyd & Dreksler, 1979; Zukin *et al.*, 1974). The antagonist bicuculline is also able to displace [³H]GABA. On the other hand, picrotoxinin, which is presumed to interact at or near the ionophore site on the receptor complex (Simmonds, 1980*a*; Olsen *et al.*, 1979), does not displace bound [³H]GABA. Compounds that interact with the transport protein for GABA (e.g. nipecotic acid or 2,4-diaminobutyric acid) also have no effect on Na^+-independent receptor binding. Subcellular fractionation studies have revealed that the majority of specific GABA binding is associated with synaptic membranes (Lester & Peck, 1979) and maximum binding is observed in the cerebellum. Autoradiographic studies using [³H]GABA have shown that, within the cerebellum, by far the majority of the receptors are localized in the granule cell layer. There is, however, some disparity between localization experiments involving radiolabelled GABA and those using muscimol (Chan-Palay, 1978). Negligible amounts of GABA binding were associated with white matter (Palacios *et al.*, 1980*a*). Additional support for the fact that binding measured *in vitro* reflects binding to functional GABA receptors comes from an examination of changes in GABA binding induced by experimental or pathological damage to the brain. Significant alterations in binding may be observed in patients with neurological disorders of motor function such as Parkinson's disease (Lloyd *et al.*, 1977*b*). Viral infections (Simantov *et al.*, 1976) or mutations (Olsen & Mikoshiba, 1978) that affect cerebellar granule cells also give rise to an apparent decrease in measurable GABA receptors.

Initial studies on the kinetics of [³H]GABA binding reported a homogeneous class of receptor sites with a dissociation constant of approx. 350 nM (Enna & Snyder, 1975). Subsequent studies have shown that this single population of sites is observed

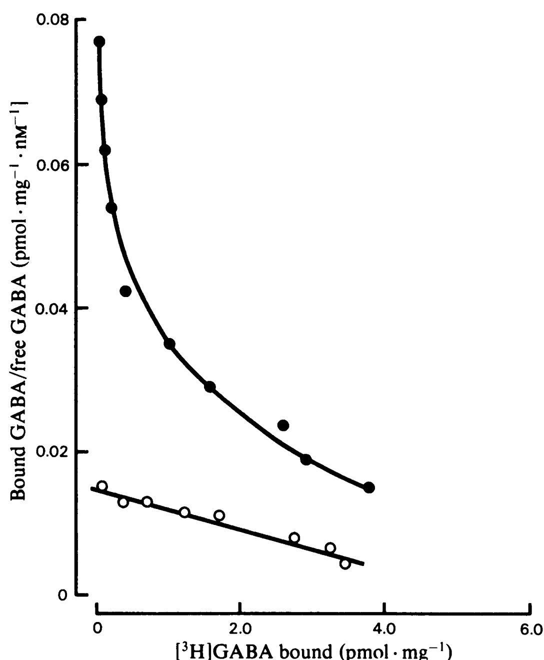

Fig. 3. *Scatchard analysis of [^{3}H]GABA binding to synaptic membranes*

Binding of [^{3}H]GABA to membranes from rat cerebral cortex before (O) and after (●) overnight dialysis was measured by a centrifugation binding assay (Greenlee *et al.*, 1978). For further details see Whittle & Turner (1982*a*). Dialysis removes endogenous inhibitors and reveals a high-affinity binding site for GABA.

in fresh tissue preparations but more complex kinetics are observed if the membrane fraction is subjected either to freeze-thawing followed by extensive washing or to treatment with low concentrations (<0.01% w/v) of Triton X-100 (Greenlee *et al.*, 1978; Toffano *et al.*, 1978; Lester & Peck, 1979; Napias *et al.*, 1980). These treatments apparently reveal a second class of receptors of approx. 10-fold higher affinity for GABA. Thorough dialysis of fresh synaptic membrane preparations have also been shown to reveal sites of 'high' (K_D = 15 nM) and 'low' (K_D = 150 nM) affinity for GABA (Whittle & Turner, 1982*a*) (Fig. 3). These two populations have been observed in several mammalian species, including humans (Olsen *et al.*, 1981*a*; Van Ness & Olsen, 1979). Although these two sites are very similar in terms of their pharmacology, they appear to represent distinct and separate entities. The proportion of the two sites varies with brain region (Van Ness & Olsen, 1979; Enna *et al.*, 1979) and they differ in their responses to pH, temperature (Olsen, 1980) and the chaotropic agent thiocyanate (Browner *et al.*, 1981). Proteolysis does not seem to account for the observed kinetic changes, since membranes prepared in the presence of a 'cocktail' of proteinase inhibitors show similar proportions of the high- and low-affinity sites (S. R. Whittle & A. J. Turner, unpublished work). Efforts to interconvert the two populations of sites have been unsuccessful (Olsen *et al.*, 1981*a*). There appears to be no co-operativity between the two sites (Horng & Wong, 1979) although electrophysiological studies have suggested that at least two molecules of GABA may be required to bind and activate chloride transport (Macdonald & Young, 1981).

The functional importance of each of the two sites detected by direct binding studies is unclear but there has been the common assumption in the literature that the cryptic site, because of its higher affinity for GABA, may be more relevant physiologically. This viewpoint may be unjustified since the conditions required to reveal the cryptic site are disruptive to membrane organization and may therefore reflect changes in the environment of the receptor. Kinetic studies under these conditions may therefore produce a misleading impression of receptor affinity *in vivo*. Since the concentration of GABA needed to activate Cl^- channels in cultured neurons is in excess of 10 μM (Barker & Mathers, 1981), a receptor site of exceptionally high affinity may not be required to mediate this process.

The observation that extensive manipulation of the membrane preparation was required to reveal the cryptic, high-affinity receptor had led to the hypothesis that endogenous inhibitors of [^{3}H]GABA binding may normally mask this site (Toffano *et al.*, 1978). If this were the case, it would have considerable impact for the regulation of synaptic activity. Claims to detect such inhibitors, though, have been varied and controversial (see e.g. Napias *et al.*, 1980). Much of the inhibitory material present in membrane preparations may be attributable to endogenous GABA, which is extremely difficult to remove (Napias *et al.*, 1980; Gardner *et al.*, 1981). Other low-M_r (<500) inhibitors chromatographically separable from GABA have also been detected in membrane preparations (Yoneda & Kuriyama, 1980). Most interest and controversy, though, has centred on peptide modulators of GABA receptor function. The largest of these, with an M_r of 15 000, has been termed GABA-modulin (Toffano *et al.*, 1978). Costa and collaborators (Massotti *et al.*, 1981) reported the purification of this protein to homogeneity and a good deal of its molecular properties have been described. GABA-modulin is postulated to reduce the number of available high-affinity sites for GABA by a mechanism that can be modulated by benzodiazepines (Guidotti *et al.*, 1979). Other groups, though, have been

unable to purify the protein, perhaps because of its reported lability, and are more sceptical of a role for this protein in GABA function (Olsen *et al.*, 1980; Napias *et al.*, 1980). Other smaller peptides that inhibit GABA binding have also been demonstrated in detergent extracts of brain membranes (Johnston & Kennedy, 1979). The possible regulation of the inhibitory GABA synapse through the action of endogenous modulators is undoubtedly an area that requires detailed clarification.

Benzodiazepines allosterically modulate the $GABA_A$ receptor

The benzodiazepine class of tranquillizers, of which diazepam (Valium) represents the prototype compound (Fig. 1), are among the most widely used drugs in clinical practice. They are extremely effective in alleviating anxiety and also have uses as anticonvulsants and muscle relaxants. The first clues to their mechanism of action came from electrophysiological investigations (see, e.g., Macdonald & Barker, 1977) in which it was possible to demonstrate that the drugs potentiate the inhibitory effects of GABA in the central nervous system by increasing the frequency of opening of the chloride channel (Study & Barker, 1981). Parallel biochemical investigations led to the discovery (Braestrup & Squires, 1977; Möhler & Okada, 1977) that radiolabelled benzodiazepines bind with high affinity to specific receptor sites in synaptic membranes. The dissociation constant for diazepam is approx. 4nM. The affinities of various benzodiazepines for these sites correlate well with their clinical and electrophysiological effects, implying that these receptors are the functional sites through which benzodiazepines exert their physiological actions (Braestrup & Squires, 1978).

Although benzodiazepines are unable to displace GABA from its recognition sites, considerable evidence has now accumulated suggesting that the receptor sites for GABA and benzodiazepines can be closely associated within the membrane. The localization of benzodiazepine receptors at GABA synapses has been visualized by means of autoradiography (Möhler *et al.*, 1981). Agonists at GABA receptor sites, e.g. muscimol or GABA itself, are able to increase the affinity of the binding site for benzodiazepines, an effect seen as an enhancement in benzodiazepine binding (Tallman *et al.*, 1978; Briley & Langer, 1978; Dudai, 1979). This potentiation is antagonized by bicuculline, implying that GABA is achieving its effect through the $GABA_A$ type of receptor (Tallman *et al.*, 1978). With few exceptions (Guidotti *et al.*, 1979; Skerritt *et al.*, 1982), most workers have been unable to observe any direct effect of diazepam in assays of GABA receptor binding (Zukin *et al.*, 1974; Olsen *et al.*, 1978; Möhler & Okada, 1978; Andrews & Johnston, 1979). Diazepam is, however, able to protect GABA receptors from thermal inactivation or modification by iodoacetamide, and GABA exerts reciprocal effects on benzodiazepine sites (Gavish & Snyder, 1980).

One technique that has particular relevance to the detection of coupling between membrane proteins is that of irradiation inactivation (Kempner & Schlegel, 1979). Application of classical target theory to inactivation data allows an estimate of the molecular target size of the irradiated species. If two or more proteins are tightly coupled within the membrane, then they present a single homogeneous target to the electron beam. Such an effect is seen, for example, with receptor–adenylate cyclase complexes (Houslay *et al.*, 1977; Rodbell, 1980; Stockton & Turner, 1981). When Chang *et al.* (1981) applied this technique to brain membrane preparations, they were able to demonstrate that the benzodiazepine receptor (estimated with [^{3}H]flunitrazepam) and the GABA receptor (estimated with [^{3}H]muscimol) exhibited identical target sizes (apparent M_r 217000) which would be consistent with the existence of a membrane complex comprising both GABA and benzodiazepine receptors together with associated ionophore. Using rather different conditions, Doble & Iversen (1982) obtained an estimate for the benzodiazepine receptor of 90000–100000, which decreased to 60000–63000 if the membranes had been pre-treated with 10^{-4}M-GABA. They speculate that GABA triggers a transition of the benzodiazepine receptor from dimer to monomer which may be related to the opening of the transmembrane chloride channel. Photolabelling experiments have even implied that a tetrameric arrangement of benzodiazepine receptor subunits may be capable of existing within the membrane (Möhler *et al.*, 1980; Karobath & Supavilai, 1982). Paul *et al.* (1981), though, obtained a value of 57000 for the M_r of brain benzodiazepine receptors from irradiation inactivation experiments and failed to observe an oligomeric state of the receptor. These apparent discrepancies may reflect differences in the preparation and handling of membrane samples allowing different degrees of coupling between the various components.

Do endogenous ligands exist for the benzodiazepine receptors?

The demonstration that benzodiazepines bind with high affinity and stereospecificity to synaptic membranes has led to the proposal that natural ligands may exist in brain for these particular receptors (see, e.g., Braestrup & Nielsen, 1980). Various groups have therefore examined brain extracts for their ability to displace [^{3}H]diazepam specifically bound to synaptic membranes, with the aim of isolating a natural 'anti-anxiety' compound

(reviewed in Tallman *et al.*, 1980). Such an approach is conceptually analogous to that used for the isolation of the opiate peptides (enkephalins and endorphins). While it is clear that brain does contain compounds capable of displacing diazepam, there is no compelling evidence that any of the compounds yet isolated function as endogenous ligands. Extraction and fractionation of the inhibitory activity has demonstrated that it is partly due to the purines inosine and hypoxanthine. Nicotinamide also shows inhibitory activity. These interactions are, however, relatively weak (K_D approx. 1mM or greater compared with 4nM for diazepam) and therefore their relevance as functional ligands must be questioned. The most potent inhibitor of diazepam binding was obtained by Braestrup and collaborators by solvent extraction of human urine (Braestrup *et al.*, 1980). They isolated a compound, subsequently identified as the ethyl ester of β-carboline 3-carboxylate (β-CCE; Fig. 1), which displayed an extremely high affinity ($K_D = 4$nM) for the benzodiazepine receptor. While compounds of this general type have provided valuable information on the nature and heterogeneity of benzodiazepine receptors (Ehlert *et al.*, 1981), the compound originally isolated by Braestrup *et al.* (1980) appears to have been formed as a result of the extraction procedure itself. Although β-carboline 3-carboxylate ethyl ester has not been detected in brain or peripheral tissues, the related tetrahydro-β-carboline alkaloids can be formed from tryptamine (Pearson & Turner, 1975). Thus the natural existence of β-carbolines remains an intriguing possibility, although the tetrahydro-compounds display a much lower affinity for the benzodiazepine receptor. A mechanism for their biological formation has been proposed (Pearson & Turner, 1979) although their detection *in vivo* requires scrupulous care in view of the ease with which tryptophan and tryptamine condense with aldehydes to generate β-carbolines.

Other potential candidates as endogenous ligands have also been isolated. These include peptides of various sizes (Massotti *et al.*, 1981; Davis & Cohen, 1980; Woolf & Nixon, 1981) as well as thromboxane A_2 and prostaglandins of the A class (Ally *et al.*, 1978; Asano & Ogasawara, 1981). Convincing pharmacological or physiological evidence that any of these compounds have a functional role in potentiating inhibitory neurotransmission is lacking. If an endogenous ligand does exist for benzodiazepine receptors, it would provide the best-described example in which activation of one receptor can directly modulate the activation of a second receptor in the cell. The less dramatic possibility must remain that the benzodiazepine receptor protein merely represents a drug-binding site on the GABA receptor complex for which no natural ligand exists.

Benzodiazepine receptors are heterogeneous

The kinetics of benzodiazepine binding to brain membrane preparations are dependent on the radiolabelled ligand that is selected. [^{3}H]Diazepam apparently binds to a single class of benzodiazepine recognition sites and the displacement of these ligands by other benzodiazepines produces no evidence of heterogeneity (Braestrup & Squires, 1977; Möhler & Okada, 1977). A Hill coefficient close to 1 is observed in this instance. However, displacement of [^{3}H]benzodiazepines by triazolopyridazine tranquillizers (Squires *et al.*, 1979) or β-carbolines (Fig. 1) (Nielsen *et al.*, 1981) produces shallow dose–response curves, implying that these ligands can differentiate between discrete classes of benzodiazepine receptors. [^{3}H]β-Carbolines have now become invaluable tools to discriminate between receptor types (Ehlert *et al.*, 1981; Hirsch *et al.*, 1982; Braestrup & Nielsen, 1980). There is general agreement that two major classes of receptor type can be distinguished, and these have been referred to as Bz_1 and Bz_2 (or type I and II). An additional 'super-high-affinity' site selective for propyl-β-carboline-3-carboxylate has also been postulated (Ehlert *et al.*, 1981). The Bz_1 sites show a 10-fold higher affinity for β-carbolines and triazolopyridazines than the Bz_2 sites. Benzodiazepines such as diazepam show very similar affinities for the two sites and are therefore unable to distinguish between them.

The kinetic evidence for heterogeneity of benzodiazepine receptors is supported by other criteria. The differential solubilization of two subtypes of benzodiazepine receptor has been reported (Lo *et al.*, 1982). In addition, the photosensitive benzodiazepines flunitrazepam and clonazepam (Möhler *et al.*, 1980; Johnson & Yamamura, 1979) can be used to label covalently benzodiazepine receptors. [^{3}H]Flunitrazepam was shown to bind irreversibly to a membrane polypeptide of apparent M_r 51000 after exposure to u.v. light (Sieghart & Karobath, 1980). In several brain regions up to three additional proteins with M_r values in the range 53000–59000 were also photolabelled. The binding of [^{3}H]-flunitrazepam to all these proteins was inhibited by diazepam and was potentiated by GABA, implying that the ^{3}H-labelled polypeptides were originally constituents of the GABA and benzodiazepine receptor complex. The size of the polypeptides is consistent with the minimum value obtained by irradiation inactivation (Doble & Iversen, 1982; Paul *et al.*, 1981) and leads to the conclusion that the benzodiazepine receptors are discrete proteins within the receptor complex.

In addition to the sites described above, benzodiazepines bind specifically to a distinct class of receptor sites in kidney and other tissues. These sites differ considerably from the Bz_1 and Bz_2 receptors in

their pharmacological selectivity (Braestrup & Squires, 1977). Unfortunately the kidney receptors for benzodiazepines have been labelled 'peripheral' sites although they are now also known to occur in brain tissue (Schoenmaker *et al.*, 1981). The function of these proteins, either centrally or peripherally, is unknown. They do not appear to be involved in the tranquillizing actions of benzodiazepines since there is no correlation between affinity of benzodiazepines for the 'peripheral' sites and their clinical potencies.

It is quite clear that GABA and benzodiazepine receptors can be coupled within the post-synaptic membrane to form a functional complex regulating chloride permeability. Deoxycholate can solubilize both [^{3}H]muscimol and [^{3}H]flunitrazepam binding activities, which migrate together when subjected to gel filtration or sucrose-density-gradient centrifugation (Stephenson *et al.*, 1982). GABA retains the ability to stimulate the binding of [^{3}H]diazepam even after solubilization and extensive purification of the benzodiazepine binding site (Martini *et al.*, 1982). However, the heterogeneity of detectable GABA and benzodiazepine receptors raises two important issues. Are all the benzodiazepine sites linked to GABA receptors and which of the kinetically detectable GABA sites is responsible for the coupling?

These two questions are inter-related since, paradoxically, it seems that the high-affinity GABA receptor may not be associated with benzodiazepine sites. When [^{3}H]muscimol is used for the detection of GABA receptors in cerebellum by autoradiography, the majority of the labelling is seen on granule cells (Palacios *et al.*, 1980*a*) whereas benzodiazepine sites are concentrated in the molecular cell layer (Young & Kuhar, 1979; Unnerstall *et al.*, 1981). Ontogenetic studies reveal that the development of GABA-enhanced benzodiazepine binding parallels the development of benzodiazepine receptors themselves rather than the increase in total [^{3}H]GABA binding (Mallorga *et al.*, 1980; Palacios *et al.*, 1980*b*). The chaotropic agent thiocyanate can selectively inactivate the high-affinity GABA receptor yet not affect the stimulation of benzodiazepine binding by GABA (Browner *et al.*, 1981). Alkylating or thiol reagents can also distinguish between these two processes (Marangos & Martino, 1981). Another anomaly is that the concentration of GABA required to produce half-maximal enhancement of benzodiazepine binding (approx. 1 μM) (Tallman *et al.*, 1978; Karobath & Sperk, 1979) is considerably greater than the measured dissociation constants for either the high- or low-affinity GABA receptors (see above). Furthermore, the relative effects of analogues of GABA on benzodiazepine binding do not parallel their action at the GABA receptor itself (Braestrup *et al.*, 1979). Finally, the difficulties encountered by many groups in demonstrating an enhancement of GABA binding by diazepam point to some differences between the kinetically detectable GABA receptors and the GABA recognition site that interacts with benzodiazepine receptors.

These differences may not be real, in the sense that they may reflect an interaction of GABA with different conformations or states of coupling of the receptor complex. However, an alternative explanation that has been proposed is that the GABA receptor coupled to the benzodiazepine sites is a novel receptor of low affinity, not normally detectable by conventional receptor-binding assays (Karobath *et al.*, 1979). The potentiation of GABA binding by barbiturates, which occurs by a mechanism different from that caused by the benzodiazepines (Whittle & Turner, 1982*a*) has also been interpreted in terms of a low-affinity class of GABA receptor (Olsen, 1981). Little concrete evidence has existed to support this hypothesis. A recent report, however, has observed such a site for GABA in fresh membrane preparations from rat brain (Skerritt *et al.*, 1982). The dissociation constant for this site (0.82 μM) is comparable with that required to potentiate benzodiazepine binding, but is substantially decreased in the presence of 100 nM-diazepam. Falch & Krogsgaard-Larsen (1982) have also interpreted their recent kinetic data in terms of three rather than two binding sites for GABA, although they were unable to characterize the site of lowest affinity satisfactorily. Whether we are dealing with multiple receptor proteins for GABA, or different conformations of a single protein modulated by the membrane environment and allosteric interactions with benzodiazepines, will ultimately require the purification and reconstitution of the individual components of the receptor complex. The anomalies discussed above do, though, highlight the problems of interpretation of receptor-binding studies when no ready biological response to receptor activation (such as adenylate cyclase activity) is measurable.

The lack of correlation between the high-affinity GABA site and benzodiazepine receptors implies that some GABA receptors are not linked to benzodiazepine receptors. Furthermore, the density of benzodiazepine receptors in brain is approx. one-third that of GABA sites. Thus the benzodiazepine binding protein itself cannot constitute the ionophore, although it may be closely associated with it (Simmonds, 1980*a*,*b*; Costa *et al.*, 1979). The converse question, whether all benzodiazepine sites are coupled to GABA receptors, is currently unresolved. Differential solubilization of benzodiazepine and GABA binding sites can be achieved with judicious choice of detergent (Greenlee & Olsen, 1979; Olsen, 1980; Guidotti *et al.*, 1980), but

this does not necessarily imply that the proteins were unassociated within the intact membrane. The observation that the binding of β-carbolines, unlike the benzodiazepines, is not enhanced by GABA (Patel *et al.*, 1981) has been interpreted as evidence for uncoupled benzodiazepine sites. However, the β-carbolines show opposite physiological effects to the benzodiazepines (Ehlert *et al.*, 1981) and therefore may preferentially bind to a different (antagonist?) conformation of the benzodiazepine receptor which is unresponsive to GABA. The 'peripheral' class of benzodiazepine receptor is most probably unrelated to the post-synaptic receptor complex for GABA (Patel & Marangos, 1982).

Anaesthetic barbiturates also potentiate GABA-mediated inhibition

Benzodiazepines are by no means the only class of drugs able to potentiate the post-synaptic action of GABA. For example, the pyrazolopyridine class of tranquillizers (e.g. Etazolate) and even ethanol can achieve this effect. Most attention, though, has focused on the action of barbiturates, particularly those with anaesthetic activity (e.g. pentobarbital). Electrophysiological evidence has implied that this class of barbiturates acts by a different mechanism from benzodiazepines, since they prolong the lifetime of the activated chloride channel (Huang & Barker, 1980). Barbiturates may therefore provide a useful probe for other components of the receptor complex. Pentobarbital displaces neither [^{3}H]diazepam nor [^{3}H]GABA from their respective binding sites at physiologically relevant concentrations. The drug is, though, able to modulate the receptor interactions of these two radioligands. Pentobarbital was first shown to increase significantly the affinity of [^{3}H]diazepam for its receptor (Leeb-Lundberg *et al.*, 1980; Skolnick *et al.*, 1981; Ticku, 1981) and this effect was blocked by picrotoxinin. Attempts to show a related effect of pentobarbital on the specific binding of [^{3}H]GABA have proved more difficult to reproduce. Barbiturates have been variously reported to have no effect on sodium-independent GABA binding (Enna & Snyder, 1976; Peck *et al.*, 1976), to increase the affinity of GABA for its high-affinity binding site (Willow & Johnston, 1980, 1981) or to increase the apparent number of detectable sites in the membrane (Olsen *et al.*, 1981*b*; Asano & Ogasawara, 1981; Whittle & Turner, 1982*a*). It is now clear that these discrepancies are principally due to the various methods of membrane preparation employed (Whittle & Turner, 1982*a*), since integrity of the receptor complex is required to observe potentiation of GABA binding by barbiturates. Activation is observed at concentrations above 25 μM. As with enhancement of benzodiazepine binding (Leeb-Lundberg *et al.*, 1980), the effect is blocked by picrotoxinin and, interestingly, the presence of chloride or other anions (e.g. I^-, Br^-, ClO_4^-, SCN^-) was required to observe the effect (Olsen *et al.*, 1981*b*). Since the anion specificity for enhancement of binding parallels the ability of these anions to permeate the picrotoxinin-sensitive chloride channel, it has been concluded that these barbiturates bind at or near the ionophore component of the receptor complex and allosterically modulate the binding of both GABA and benzodiazepines (Olsen, 1981). ^{3}H-labelled dihydropicrotoxinin has been used as a ligand to examine these interactions in more detail (Ticku *et al.*, 1978*a,b*). This convulsant toxin is not directly displaced by GABA or benzodiazepines, implying that it binds to a discrete component of the receptor complex, consistent with an effect at or near the ion channel (Olsen, 1981). The ability of pentobarbital to inhibit the binding of dihydropicrotoxinin has been interpreted in terms of a 'picrotoxinin/barbiturate receptor' associated with the GABA receptor and, perhaps, comprising part of the ionophore. Subsequently, other convulsant or depressant drugs have been postulated, like barbiturates, to act through this component on the basis of their ability to inhibit picrotoxinin binding (Ticku & Olsen, 1978). [^{3}H]-Phenobarbital has recently been reported to bind to synaptic membranes, although with low affinity (K_D approx. 100 μM) and at a density far in excess of the number of GABA receptor sites (Willow *et al.*, 1981). Several lines of evidence raise some doubts, though, about the concept of a specific membrane receptor site for barbiturates. Conclusions drawn from experiments on [^{3}H]dihydropicrotoxinin binding must be viewed with some caution, since the toxin exhibits a high degree of non-specific binding to membrane preparations (Ticku & Olsen, 1978; Ticku *et al.*, 1978*a*). In addition, relatively high concentrations of barbiturates (compared with those of benzodiazepines) are required to observe effects. At these concentrations, barbiturates can modify the lipid environment of membrane proteins. Indeed, Seeman (1972) proposed that barbiturates may exert their depressant effects within the nervous system by such a mechanism. A specific example is provided by the ability of phenobarbital to modulate hormone-stimulated adenylate cyclase activity through a selective fluidization of the outer face of the membrane bilayer (Houslay *et al.*, 1981). It is therefore possible that anaesthetic barbiturates modify the kinetic behaviour and state of coupling of the GABA receptor complex, at least in part, through changes in membrane fluidity. If so, the anion specificity of the barbiturate activation process may relate more to the chaotropic properties of these ions (e.g. ClO_4^-, SCN^-) rather than their ability to permeate the chloride channel. Phospholipids are well known to affect binding at the GABA recognition site (Giambalvo & Rosenberg, 1976;

Johnston & Kennedy, 1978) and perturbation of the lipid environment of the receptor complex may play a greater role in its regulation by drugs than previously supposed. Clearly this is an area for more detailed investigation which may help to explain the ability of ethanol to potentiate GABA-mediated inhibition, since this alcohol can also modify the fluidity of neuronal membranes (Goldstein *et al.*, 1982).

Presynaptic receptors for GABA regulate transmitter release

There are now many precedents for the existence of multiple classes of receptors for neurotransmitters (Snyder & Goodman, 1980). Sub-classes of receptors may mediate their physiological responses through different membrane transduction systems (cf. the α- and β-classes of adrenergic receptors). It is therefore not surprising that receptor populations for GABA can be subdivided, although no wholly satisfactory classification system yet exists. The discussion to date has focused on the post-synaptic GABA receptors that are sensitive to bicuculline. It is this population of receptors that has been the most clearly defined in molecular terms. However, GABA receptors located pre-synaptically have also been detected *in vitro* and these may be further subdivided depending on their sensitivity to bicuculline (Table 1).

In general, pre-synaptic receptors regulate the release of neurotransmitter through a negative feedback mechanism (Starke, 1981). Thus GABA may inhibit its own release from pre-synaptic terminals (Mitchell & Martin, 1978) (through 'autoreceptors') or modulate the release of other transmitters (through 'heteroreceptors'). The regulation of GABA release by autoreceptors has been demonstrated by using muscimol, which mimics many of the actions of GABA. Muscimol inhibits the K^+-dependent release of [^{3}H]GABA previously accumulated by brain synaptosomes. This effect is opposed by bicuculline and picrotoxinin but does not appear to be modulated by benzodiazepines (Brennan, 1982). Interestingly, the porphyrin precursor δ-aminolaevulinic acid may be a selective agonist at these autoreceptors (Brennan & Cantrill, 1979).

Several classes of GABA heteroreceptors have been suggested, principally from transmitter release studies. GABA is able to stimulate the release of [^{3}H]glutamate from slices of striatum, through a pre-synaptic receptor on nerve terminals that employ glutamate as excitatory transmitter (Mitchell, 1980*a*, 1982). This pre-synaptic receptor is sensitive to bicuculline and picrotoxinin and is modulated by benzodiazepines (Mitchell, 1980*b*). However it shows a different order of potency of GABA agonists (3-aminopropanesulphonate > isoguvacine > muscimol) compared with the 'classical' post-synaptic $GABA_A$ receptor (Mitchell, 1982).

Perhaps the most exciting development has come from studies with Baclofen [4-amino-3-(4-chlorophenyl)butanoic acid; Fig. 1]. This drug was originally developed as a hydrophobic analogue of GABA capable of crossing the blood-brain barrier. The first clues to its novel action came from an examination of the effects of GABA and Baclofen on the evoked release of [^{3}H]noradrenaline from cerebellar slices (Bowery *et al.*, 1980) and from certain peripheral nerve terminals (Bowery *et al.*, 1981*a*; Muhyaddin *et al.*, 1982). Both compounds inhibited the release process, yet their action was not antagonized by bicuculline or picrotoxinin, nor modulated by benzodiazepines (Bowery *et al.*, 1981*a*; Muhyaddin *et al.*, 1982). In fact, the specificity of bicuculline as an antagonist of GABA action had been questioned more than 10 years previously (Godfraind *et al.*, 1970). Baclofen may also act pre-synaptically to block the release of excitatory amino acid transmitters, perhaps by a different mechanism (Ault & Evans, 1981). Radioligand binding studies using [^{3}H]Baclofen have confirmed the presence of a population of bicuculline-insensitive GABA receptors (Hill & Bowery, 1981). The hydrophobic nature of Baclofen, however, makes it a less than ideal ligand for this purpose, since it readily partitions into the membrane lipid bilayer. The binding of Baclofen was shown to be stereospecific and revealed a single class of sites with a dissociation constant of approx. 130nM (Hill & Bowery, 1981). These sites have now been referred to as $GABA_B$ receptors and their concentration in various brain regions is approx. 25% of that of the $GABA_A$ receptors. The cerebellum contains the highest concentration of $GABA_B$ sites, where autoradiographic studies have shown that they predominate in the molecular rather than granule cell layer (Wilkin *et al.*, 1981). The reverse is true of the bicuculline-sensitive sites. The precise cell types on which the $GABA_B$ sites are located is unclear, although they do not appear to be associated with glial cells. The release studies (Bowery *et al.*, 1980) suggest that they are present on pre-synaptic nerve terminals where they modulate transmitter release.

Functionally the $GABA_B$ sites appear very distinct from the well-characterized $GABA_A$ receptors. An essential requirement for their detection by direct binding studies was the inclusion of divalent cations (typically Ca^{2+}). Another characteristic of the $GABA_B$ receptor is that it is influenced by guanyl nucleotides which decrease the affinity of binding of GABA agonists (Bowery *et al.*, 1981*b*). Such regulation is a feature of many hormone and neurotransmitter receptors that interact with adenylate cyclase, either through activation or inhibition of

the enzyme. The latter event is a possible outcome of receptor activation at $GABA_B$ sites, but this has not been directly demonstrated. The ultimate effect is suggested to be a decrease in the inward flux of Ca^{2+} into the pre-synaptic nerve terminal, thereby inhibiting the release of neurotransmitter (Hill & Bowery, 1981; Dunlap, 1981).

Thus the two major classes of GABA receptor (bicuculline-sensitive A and bicuculline-insensitive B) would appear to function through entirely separate signalling mechanisms. In this respect there is a striking parallel with acetylcholine receptor mechanisms. Activation of one class of receptor results in a rapid trans-membrane response through direct activation of an associated ionophore (transporting Na^+ at nicotinic cholinergic receptor or Cl^- at the $GABA_A$ receptor). The second class of receptors is modulated by guanyl nucleotides and their effects may be mediated by changes in cyclase activity, Ca^{2+} flux or other unidentified mechanisms (muscarinic cholinergic and $GABA_B$ receptors).

The ability of Baclofen to inhibit catecholamine release is an effect also seen with 4-hydroxybutyrate (γ-hydroxybutyrate, GHB) (Da Prada & Keller, 1976). This naturally-occurring metabolite of GABA has been identified in brain and has been shown to mimic the actions of GABA in some experimental systems (Giorguieff *et al.*, 1978; Unnerstall & Pizzi, 1981). However, it has not proved possible to show an effect of 4-hydroxybutyrate on either $GABA_A$ (Lloyd & Dreksler, 1979) or $GABA_B$ (Hill & Bowery, 1981) sites in receptor-binding studies.

The most clearcut response to administered 4-hydroxybutyrate is an increase in the levels of dopamine in the brain, which has been ascribed to a decreased firing rate of dopamine neurons. The increase in dopamine results from a kinetic activation of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis (Morgenroth *et al.*, 1976). This effect on the release and synthesis of dopamine is presumed to occur through an interaction with specific pre-synaptic receptors (Stock *et al.*, 1973) and appears to be sensitive to picrotoxinin (Roth & Nowycky, 1977).

Two distinct possibilities emerge. 4-Hydroxybutyrate may be interacting with a sub-class of GABA receptors sited pre-synaptic to dopamine neurons. Alternatively, and more controversial, is the suggestion that 4-hydroxybutyrate may itself function in a neurotransmitter capacity. Foremost in proposing the latter possibility have been Mandel and colleagues (see e.g. Rumigny *et al.*, 1980, 1981). A number of criteria for such a role have apparently been satisfied. Several groups have been able to demonstrate the existence in brain of enzymes capable of synthesizing 4-hydroxybutyrate (Hoffmann *et al.*, 1980; Rivett *et al.*, 1981; Whittle & Turner, 1982*b*). Curiously, the reductase(s) responsible for its production from succinic semialdehyde are cytosolic in location, whereas GABA transaminase is mitochondrial (Rumigny *et al.*, 1981). Thus translocation of the aldehyde across the mitochondrial membrane must be postulated to occur before its reduction. A similar compartmentation problem exists, though, for the reductive metabolism of catecholamines (Ryle & Tipton, 1981; Turner *et al.*, 1982). Other criteria supporting a functional role for 4-hydroxybutyrate have been less clearly defined. However, a specific transport system has been demonstrated in synaptic membrane preparations which may function in its removal from the synapse (Benavides *et al.*, 1982*a*). More significantly, it is reported by the same group (Benavides *et al.*, 1982*b*) that a specific binding site of high affinity for 4-hydroxybutyrate occurs in synaptic membranes which is distinct from the transport site. This may represent the biochemical counterpart of the pre-synaptic receptor postulated to modulate dopamine function. If so, then it is distinct from any known class of GABA receptor, since 4-hydroxybutyrate is not displaced by GABA or Baclofen, even at high concentration (Benavides *et al.*, 1982*b*). Despite these observations, the status of 4-hydroxybutyrate in neuronal function must be regarded as questionable at present. Much of this metabolite may be localized in glial cells and the concentrations present in the region of dopamine neurons may be inadequate *in vivo* under normal conditions to modify the nerve impulse (Roth *et al.*, 1980). Furthermore, although an uptake system has been demonstrated for 4-hydroxybutyrate, there is no defined mechanism for its further metabolism and inactivation. The possibility remains, therefore, that it is merely mimicking the action of GABA through an ill-defined population of GABA receptors (Olpe & Koella, 1979). 4-Hydroxybutyrate will continue to prove useful experimentally for investigations of pre-synaptic receptor mechanisms which are crucial to the homeostatic regulation of the neuron (Starke, 1981).

Conclusions

In conclusion, this review has attempted to summarize the varied mechanisms through which GABA exerts its central inhibitory actions. The contributions of neurophysiologists and pharmacologists have laid the foundations for understanding how these processes are mediated at the biochemical level. Indeed, the discussion has highlighted some of the limitations of receptor-binding studies when used in isolation. The availability of more selective drugs for the various classes of detectable GABA receptors will be essential both for effective clinical therapy and for defining the location and function of

these receptors. It will also be a prerequisite for a more systematic classification of the various receptor types. Most investigations have centred upon the post-synaptic receptor complex for GABA, and the solubilization and purification of the components of this complex is well advanced in a number of laboratories. Reconstitution of these components in model membranes will be required in order to define the stoichiometry and organization of the various polypeptides needed for activation of Cl^- conductance in response to GABA. A number of intriguing questions remain unresolved. Is the ionophore composed of polypeptide chains distinct from the GABA and benzodiazepine receptor sites? Does the membrane environment play an important role in modulating the activity of the receptor? How is the coupling of the receptor components effected? Do endogenous ligands regulate the benzodiazepine receptor and hence potentiate or inhibit the action of GABA? Opinion is divided on each of these issues. Future studies will require the availability of more specific probes for the identifiable receptor components. The development of monoclonal antibodies to the receptor complex would therefore provide a major advance in this direction. Ultimately, a molecular description of the organization and regulation of the inhibitory synapse will have considerable relevance to membrane transduction processes in general.

Note added in proof

Since this review was written, a detailed account of the purification and characterization of GABA-modulin has appeared (Guidotti *et al.*, 1982). The possible regulation of GABA receptor sites by GABA-modulin is discussed therein.

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