Psychopharmacology (1984) 83:304–315

Psychopharmacology

Review

Multiple sites of action for anxiogenic drugs: Behavioural, electrophysiological and biochemical correlations

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Abstract. This review describes animal models of anxiety that are able to identify an anxiogenic drug effect. Evidence is reviewed for the anxiogenic action of several drugs that act at the GABA-benzodiazepine-chloride ionophore complex in the brain. The effects of their combinations with various other drugs thought to act at the same sites are discussed. The classification of these drugs on the basis of their behavioural profiles is compared with their classification based on biochemical and electrophysiological studies.

Key words: Anxiety – Social interaction – Conflict – Drug discrimination – Ro 15-1788 – CGS 8216 – β-carbolines – Ro 5-4864 – Ro 5-3663 – Picrotoxin – Pentylenetetrazol – Benzodiazepine receptors – Picrotoxinin site

The benzodiazepines are widely prescribed and clinically effective anxiolytics that are thought to produce their pharmacological actions via specific high affinity binding sites in the brain (Squires and Braestrup 1977; Möhler and Okada 1977). The later discovery of drugs that specifically antagonise the actions of benzodiazepines (Hunkeler et al. 1981; Yokoyama et al. 1982; Braestrup et al. 1980) has provided a new set of tools with which to elucidate their mechanisms of action. These new compounds are of particular interest in the light of recent evidence that they also have intrinsic actions, generally in the opposite direction to those of benzodiazepines. The discovery that the these drugs are anxiogenic in several animal tests of anxiety (File et al. 1982a; File and Lister 1983a; Mendelson et al. 1983; Corda et al. 1983; Prado de Carvalho et al. 1983a) and in man (Dorow et al. 1983) is important for investigating the neural substrates of anxiety. This review concentrates on the anxiogenic actions of drugs that are thought to act via benzodiazepine binding sites or related sites (e.g. the picrotoxinin site). This is not to suggest that these are the only sites capable of mediating anxiety: over the years several compounds have been reported to have anxiogenic effects, and their sites of action may be quite different from the GABA-benzodiazepine-ionophore complex considered in this review. These include caffeine, amphetamine (File and Hyde 1979) and yohimbine (Holmberg and Gershon 1961; Lal et al. 1983). However, recent evidence that caffeine may act at the benzodiazepine site to cause seizures (Velluci and Webster 1984) and the fact that yohimbine is a β-carboline derivative suggest that these compounds may also produce their anxiogenic actions via this complex. However, these anxiogenic actions are not sufficiently well characterised to warrant inclusion here.

Table 1 provides a brief summary of the known sites of action of the drugs to be discussed in this review. For further detail, see references provided.

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1. Animal tests of anxiogenic drug effects

Prado de Carvalho et al. (1983a) recently modified the *Geller-Seifter procedure* (Geller and Seifter 1960) to enable an anxiogenic drug action to be detected in mice. Mice are deprived of food and trained to lever-press for food reward. During the first and last 5-min periods in a 15-min session, lever-pressing under a CRF schedule produces a food pellet (non-conflict period). During the central 5-min period, responding (also under a CRF schedule) produces a pellet and concomitant foot shock (conflict period). An anxiogenic drug effect would be indicated by a specific decrease in rate of responding in the central 5-min period (i.e. without an effect on unpunished responding). It was necessary to use mice with high lever-pressing rates during the conflict period to see a clear reduction.

In the *Vogel punished drinking test* (Vogel et al. 1971), rats that have been water-deprived are given shocks while drinking: anxiolytic benzodiazepines increase the number of shocks taken. In order to detect an anxiogenic action (i.e. a decrease in the number of shocks taken during the session), Petersen et al. (1983) and Corda et al. (1983) lowered the shock intensity so that control animals made a high number of licks.

Because anxiolytics increase food and fluid consumption (see Cooper 1983) and the effects of most anxiogenic drugs on appetite have not been investigated, it is essential to include unpunished periods in these tests to identify such drug effects. Any drug effects on pain threshold will selectively affect an animal's responding on punished schedules and be a confounding factor. In addition, the specificity of both of these tests can be questioned on the basis of the false positives that they have produced for anxiolytic effects (e.g. see Beer et al. 1972); the same may be true for anxiogenic effects.

Since in conflict procedures the animal receives a specific punishment at a predictable time and place, these tests could be considered as tests of conditioned fear, and the relationship between conditoned fear and anxiety is uncertain. In contrast the *social interaction test* (File and Hyde 1978; File 1980) relies on the uncertainty generated by unfamiliar

Table 1. A summary of known binding sites for anxiogenic drugs

Site	Affinity for BDZ	Affinity for Ro 5-4864	Other drugs that bind	Compounds that affect binding	Other information
Classical CNS benzodiazepine (BDZ) site (Squires and Braestrup 1977; Mohler and Okada 1977)	High, e.g. 5 nM for clonazepam (Braestrup and Squires 1977)	Low: 163 μM (Braestrup and Squires 1977)	β-Carbolines (Braestrup et al. 1980); Ro 15-1788 (Hunkeler et al. 1981); CGS 8216 (Czernik et al. 1982)	GABA/muscimol (Tallman et al. 1978); chloride anions (Costa et al. 1979); barbiturates (Leeb-Lundberg et al. 1980)	Binding of BDZs correlates with their behavioural potency (Braestrup and Squires 1978); Heterogeneity of sites – see section II (b)
Peripheral-type BDZ site (a) periphery (Braestrup and Squires 1977); (b) in brain (Schoemaker et al. 1981)	High, but low for clonazepam: 3–8 μM (Braestrup and Squires 1977)	High: 4 nM (Braestrup and Squires 1977)	PK 11195 (LeFur et al. 1983a, b); NOT-β-carbolines (Braestrup and Nielsen 1983); Ro 15-1788 (Richards et al. 1982); CGS 8216 (Czernik et al. 1982)	No effect of GABA, chloride anions, picrotoxin or barbiturates (Marangos et al. 1982; Schoemaker et al. 1983)	Predominantly glial localisation (Schoemaker et al. 1983); functional relevance unknown
Micromolar BDZ site (Bowling and DeLorenzo 1982)	Micromolar affinity (Bowling and DeLorenzo 1982)	Equipotent with BDZs (Bowling and DeLorenzo 1982)	Phenytoin (Bowling and DeLorenzo 1982)	No effect of GABA (Bowling and DeLorenzo 1982)	Linked to Ca^{2+}-calmodulin kinase system (Bowling and DeLorenzo 1982) Failure to confirm (File et al. 1984b)
Dihydropicrotoxinin site (Ticku et al. 1978)	Low: micromolar affinity (Leeb-Lundberg et al. 1981)	Ro 5-4864 displaces [^{3}H]-TBPT[a] binding (20 μM, Ticku and Ramanjaneyulu 1984) but not picrotoxinin binding (Leeb-Lundberg et al. 1981)	TBPT[a] (Ramanjaneyulu and Ticku 1983); convulsants (Olsen et al. 1980; Ticku and Olsen 1979); anticonvulsants (Olsen and Leeb-Lundberg 1981); barbiturates (Ticku and Olsen 1978)		BDZ binding does not correlate with behavioural potency (Leeb-Lundberg et al. 1981) Together with GABA & BDZ sites, forms supramolecular complex in CNS (see Olsen 1982) Linked to chloride ionophore (Costa et al. 1979)

[a] [^{35}S]t-butylbicyclophosphorothionate

environments. It has proved particularly sensitive to the anxiogenic actions of drugs. In the test for detecting anxiolytic activity four different test conditions are used, but one condition is sufficient to detect an anxiogenic action. Anxiogenic agents decrease the time spent in social interaction, and this is best seen when control scores are highest, i.e. when rats are tested in low light and are familiar with the test arena. A measure of locomotor activity provides a control for drugs that decrease social interaction because of their sedative effects: a drug is unequivocally identified as anxiogenic only if the reduction in social interaction is not concomitant with a drop in locomotor activity. The advantages of this test over conflict tests are that (a) the animals are not deprived or shocked, (b) the animals' behaviour is natural, (c) no training is necessary.

A problem common to the three tests discussed above is that of confounding anxiogenic and sedative actions. The inclusion of non-conflict periods in the Geller-Seifter and Vogel tests should control adequately for sedation. However, this problem may be more severe for the social interaction test because spontaneous behaviours are more sensitive to such disruption than those in which a deprived rat is responding to obtain food or water. As will be seen in later sections, some of the drugs appear to have both anxiogenic and sedative effects. In these cases, however, we have recourse to data from tests specifically measuring sedation, and certainly the two effects are not universally confounded (e.g. File and Velluci 1978; File and Hyde 1979).

Lal and Shearman (1982) describe a *drug-discrimination procedure* that they use as a model of drug-induced anxiety. Animals are trained to press a specific lever when treated with a particular drug, and to press a different lever when treated with the drug vehicle. The perception of the drug's action by the subject serves as a cue (the interoceptive discriminative stimulus, IDS) for the selection of an appropriate response. Other drugs can then be tested for their ability to generalise to the IDS produced by the training drug: if the animal selects the drug lever rather than the saline lever, the test drug is said to generalise to the training drug, and to have a similar IDS. The drug used by Lal and co-workers to induce an anxiogenic IDS is pentylenetetrazol (PTZ), which causes intense anxiety in patients (Rodin 1958; Rodin and Calhoun 1970) and which is convulsant at higher doses. However, the specificity of the IDS in drug discrimination is always open to question. The IDS is likely to consist of an amalgam of several effects for each drug. Although the most salient cue may be the most important, it is possible that a sedative and convulsant anxiogenic drug (e.g. PTZ)

may not generalise to a non-sedative, non-convulsant (or proconvulsant) anxiogenic compound (e.g. Ro 15-1788, see section 2).

In conclusion, there are limitations with all the animal models currently in use, and there will always be some problems of interpretation. However, it should be noted that, in this respect, animal models do not differ significantly from the clinical case, where anxiety is also used as a hypothetical construct based on behavioural evidence. It is reassuring for the general validity of these models that there is considerable agreement among them in the identification of anxiogenic drugs, since each test concentrates on a different measure of anxiety. Equally reassuring are findings that certain drugs identified as anxiogenic in animal tests produce anxiety in man (see sections 2–4).

2. Drugs acting at "classical" CNS benzodiazepine sites Table 2)

a) Behavioural data

Ro 15-1788 is an imidazodiazepine that was initially characterised as a pure antagonist of benzodiazepines, lacking any intrinsic actions (Hunkeler et al. 1981). However, recent work with this compound has indicated that even at low doses it has several intrinsic actions (File et al. 1982a, b; Greksch et al. 1983) and that it becomes benzodiazepine-like at high doses (Nutt et al. 1982; Dantzer and Perio 1982; Velluci and Webster 1982; Pellow et al. 1984a).

Ro 15-1788 has been reported to antagonise the anxiolytic actions of the benzodiazepines (Hunkeler et al. 1981). When given alone, at a dose of 4–10 mg/kg, it was found to reduce active social interaction in rats without affecting locomotor activity: its effects can therefore be interpreted as anxiogenic (File et al. 1982a). This anxiogenic effect has since been replicated (File and Lister 1983b; File and Pellow 1984a). In contrast to benzodiazepines, Ro 15-1788 (8 mg/kg) accentuated the animal's response to novelty in a modified open field test: this was interpreted as an anxiogenic action (Hoffman and Britton 1983). An anxiogenic profile has not been identified for Ro 15-1788 in conflict procedures: however, it seems that a suitable dose has not yet been tried. For example, Corda et al. (1983) found that Ro 15-1788 (2 mg/kg) had no effect in the Vogel punished drinking test, but a significant effect was not seen in the social interaction test and in the open field/novelty procedure until 8–10 mg/kg. Prado de Carvalho et al. (1983a) failed to find an effect at 20 mg/kg in the Geller-Seifter conflict test, but since at 20 mg/kg Ro 15-1788 loses its anxiogenic action (File et al. 1982a), which is consistent with a benzodiazepine-like activity at higher doses, it is likely that this was too high a dose. Gherezghiher and Lal (1982) and Kehr and Stephens (1984) found that Ro 15-1788 does not generalise to the PTZ IDS in rats in doses up to 40 mg/kg. Given that the PTZ cue is likely to consist of more than just anxiety (see section 1), this failure is likely to be due to the very different behavioural profile of Ro 15-1788 in that it is neither sedative, nor convulsant or proconvulsant.

Ethyl β-carboline-3-carboxylate (*β-CCE*) was isolated from purified extracts of human urine and rat brain, and was found to have a high affinity for benzodiazepine binding sites in the CNS (Braestrup et al. 1980). It was initially thought that this compound might be an endogenous ligand for benzodiazepine binding sites, but it was later discovered to be an artefactual product of the purification procedure. There is evidence that β-CCE and other β-carbolines can reverse several of the actions of benzodiazepines, including their anxiolytic ones (Brown and Johnson 1982; Velluci and Webster 1982), and they are proconvulsant (β-CCE and β-carboline-3-carboxylate methyl amide or *FG 7142,* Oakley and Jones 1980; Jensen et al. 1983) or convulsant (methyl β-carboline-3-carboxylate or *β-CCM* and methyl 6,7-dimethyl-4-ethyl β-carboline-3-carboxylate or *DMCM,* Jones and Oakley 1981; Braestrup et al. 1982).

In the social interaction test, several β-carbolines have now been shown to have an anxiogenic action. β-CCE (1 and 2 mg/kg) reduced active social interaction in pairs of rats (File et al. 1982a). At the higher dose there was also some reduction in locomotor activity, but this was considerably less marked than the reduction in social interaction, and so is probably secondary to the anxiogenic effect. β-CCE, unlike drugs that are simply sedative, did not increase passive body contact in these rats. Also, in the hole-board test (which provides separate measures of locomotor activity and exploratory head-dipping, File and Wardill 1975), β-CCE was not sedative at these doses (File and Lister 1983c). FG 7142 (β-carboline-3-carboxylate methyl amide), another proconvulsant carboline (Jensen et al. 1983), had a specific anxiogenic action at 5 and 10 mg/kg that was significantly correlated with high plasma concentrations of the drug (File et al. 1984b). Propyl β-carboline-3-carboxylate (β-CCP) also significantly reduced time spent in active social interaction (2 mg/kg) without affecting locomotor activity, indicating an anxiogenic action (File et al. 1984a).

The anxiogenic action of β-carbolines has also been confirmed in the Vogel punished drinking test. β-CCE (0.2 and 0.4 mg/kg) induced a dose-dependent decrease in the number of shocks received by rats during a 3 min test (Corda et al. 1983), as did several other carbolines including β-CCM (0.15 mg/kg), DMCM (0.2 mg/kg) and FG 7142 (2.25 mg/kg). Similar results were obtained by Petersen et al. (1983) for β-CCE (10 mg/kg), DMCM (0.5 mg/kg) and FG 7142 (10 mg/kg). β-CCM (1 mg/kg) was also found to reduce punished responding in the Geller-Seifter conflict paradigm (Prado de Carvalho et al. 1983a), as was FG 7142 (Rossier et al. 1983). In the drug discrimination paradigm β-carbolines, including FG 7142 (ED_{50} 2 mg/kg) and DMCM (ED_{50} 0.3 mg/kg) generalise to the PTZ discriminative stimulus, indicative of an anxiogenic action (Lal et al. 1982; Kehr and Stephens 1984).

There is an observational report (Ninan et al. 1983) of increased anxiety in the rhesus monkey elicited by β-CCE (2.5 mg/kg): an acute behavioural syndrome characterised by elevations in heart rate, blood pressure, plasma cortisol and catecholamines resulted. Similarly, Ongini et al. (1983) describe a behavioural syndrome in the cat induced by FG 7142 (10 mg/kg) that they consider to be anxiety: this consists of a state of alertness, attentive behaviour and fearfulness. However, the utility of observational reports such as these can be questioned given their uncontrolled nature and the possibility of experimenter-induced bias. The anxiogenic effect of FG 7142 has also been found in man: when given to human volunteers FG 7142 produced severe anxiety attacks in cases where plasma concentrations of the drug were high (Dorow et al. 1983).

CGS 8216 is a pyrazoloquinoline that has been reported to antagonise several actions of benzodiazepines, including

their anxiolytic actions (Bernard et al. 1981; Yokoyama et al. 1982). It has intrinsic actions, including proconvulsant activity in mice (File 1983), and there is also evidence from three animal tests that this drug is anxiogenic. CGS 8216 (10 mg/kg) reduces active social interaction in rats (File and Lister 1983a, b; File and Pellow 1984c). In the Vogel conflict test, CGS 8216 reduces the number of shocks taken by rats (Mendelson et al. 1983: 5–10 mg/kg; Petersen et al. 1983: 2 mg/kg), but this effect was not seen with lower doses of CGS 8216 (1 mg/kg, Corda et al. 1983). CGS 8216 (ED_{50} 10 mg/kg) substitutes for pentylenetetrazol in a drug discrimination test (Kehr and Stephens 1984).

The interactions of these drugs in combination with each other have also been investigated. In the social interaction test, Ro 15-1788 (10 mg/kg) and β-CCE (1 mg/kg) reverse each other's anxiogenic action (File et al. 1982a), but when Ro 15-1788 or β-CCE was combined with CGS 8216 (10 mg/kg), no mutual antagonism of the drugs' effects was observed (File and Lister 1983b). Equally, there was a mutual antagonism of the anxiogenic effects of Ro 15-1788 (10 mg/kg) and FG 7142 (5 mg/kg), but not of CGS 8216 (10 mg/kg) and FG 7142 (File and Pellow 1984c). This implies a separate mechanism of action for CGS 8216 [see (b) below]. Ro 15-1788 (5 mg/kg) blocks the acute behavioural anxiety syndrome seen with β-CCE (2.5 mg/kg) in the rhesus monkey (Ninan et al. 1982), and (10 mg/kg) the behavioural syndrome produced by FG 7142 in cats (Ongini et al. 1983). Ro 15-1788 (2 mg/kg) and CGS 8216 (1 mg/kg) altered the decrease in punished licking produced by FG 7142 (4 mg/kg), DMCM (0.2 mg/kg) and β-CCM (0.15 mg/kg, Corda et al. 1983): however, the latter results with CGS 8216 contrast with those that we have obtained in the social interaction test (File and Lister 1983b; File and Pellow 1984c). The reason for this is not clear, but may lie in the different test procedures used (conflict as opposed to social interaction).

Benzodiazepines have been tested in combination with these drugs in an attempt to block their anxiogenic actions. Chronic chlordiazepoxide (5 mg/kg) reversed the anxiogenic action of Ro 15-1788 (10 mg/kg), but not of CGS 8216 (10 mg/kg, File and Pellow 1984a, File and Pellow in preparation). Given acutely, chlordiazepoxide (at 5 mg/kg) reversed the anxiogenic effect of FG 7142 (File and Pellow 1984c); at 5 or 10 mg/kg it had no effect on the anxiogenic action of Ro 15-1788 (File and Pellow 1984a), and at 5, 10 or 20 mg/kg it enhanced the reductions in time spent in active social interaction and locomotor activity seen with CGS 8216 (File and Pellow in preparation). Diazepam (1–2 mg/kg) markedly attenuated the acute behavioural anxiety syndrome caused by β-CCE (2.5 mg/kg) in rhesus monkeys (Ninan et al. 1982), and diazepam (0.5 mg/kg) antagonised the suppression of punished drinking elicited by FG 7142 (4 mg/kg), DMCM (0.2 mg/kg) or β-CCM (0.15 mg/kg, Corda et al. 1983). For a summary of these results, see Table 2.

b) Biochemical data

To date, the only high affinity binding sites that have been identified for Ro 15-1788 and β-CCE are the classical benzodiazepine CNS sites, and so the fact that these two drugs reverse each other's anxiogenic action implicates the classical CNS sites in their mediation. Braestrup and Nielsen (1983) showed that convulsant β-carbolines did not inhibit TBPT binding, and Ramanjaneyulu and Ticku (1983) report that DMCM, β-CCE and Ro 15-1788 did not interfere with the ability of convulsant or depressant drugs to inhibit TBPT binding. Also, β-CCE, β-CCM, β-CCP, Ro 15-1788 and CGS 8216 have negligible activity in inhibition of Ro 5-4864 binding to peripheral-type receptors in the brain and the periphery (Schoemaker et al. 1981, 1983; Czernik et al. 1982; Richards et al. 1982). That Ro 15-1788 can reverse both the anxiolytic action of benzodiazepines (Hunkeler et al. 1981) and the anxiogenic action of β-carbolines (File et al. 1982a; Corda et al. 1983; File and Pellow 1984c) has to be accounted for in any attempt to identify the neural substrates of anxiety. It is immediately clear that the initial conventional pharmacological account of agonist/antagonist interactions with a single binding site is an insufficient one. Recent biochemical research has provided two different models that might account for these data: the possibility of multiple classical CNS sites, and differences between ligands in the extent to which their binding is affected by GABA or barbiturates.

Initial studies with benzodiazepines suggested that they interacted with their binding sites in a simple bimolecular fashion (Squires and Braestrup 1977; Möhler and Okada 1977). However, recent studies have suggested that there exist two subpopulations of classical CNS benzodiazepine binding sites, with differential affinity for triazolopyridazines and β-CCE (Squires et al. 1979; Nielsen and Braestrup 1980). Ehlert et al. (1981) proposed that there might also exist a small proportion (3–6% of total number) of superhigh affinity sites (30–50 pM) for β-carbolines. However, for several reasons (see Martin et al. 1983) the validity of these suggestions has been questioned. It is quite clear from the evidence that the interaction of these ligands with [^{3}H]-flunitrazepam and with the benzodiazepine receptor does not follow simple mass action kinetics, and although the existence of multiple sites is a possible interpretation, this cannot at present be distinguished from other interpretations, e.g. state interconversion and receptor–receptor interactions (cooperativity, i.e. the affinity of one binding site for a ligand is affected by the presence of a ligand at some other site). What seems a more likely possibility is that there exist two (or maybe more) conformations of a single binding site. Dissociation from these conformations initiated by a benzodiazepine obeys different kinetics than when initiated by β-CCE (Doble et al. 1982; Martin and Doble 1983), also implying that the binding mechanism for β-CCE may be fundamentally different from that of the benzodiazepines.

Photoaffinity labeling studies also support the notion that there may be separate sites for β-carbolines and for benzodiazepines, and in fact current models take into account the intrinsic anxiogenic action of Ro 15-1788. Flunitrazepam, following ultraviolet radiation, is able to bind irreversibly to membrane preparations and thereby occlude benzodiazepine sites. The ability of such membrane preparations to bind benzodiazepines with the same affinity as untreated membranes is thus reduced by approximately 75% (Möhler et al. 1980; Thomas and Tallman 1981). However, there is no effect on the binding capability of photolabelled membranes for β-CCE, CGS 8216 or Ro 15-1788 (Thomas and Tallman 1983; Möhler 1982). It was suggested that there is a separate binding site on the benzodiazepine receptor complex for these three antagonists which is not occluded by photoaffinity labelling, or that

Table 2. A summary of the tests in which an anxiogenic action has been identified for drugs thought to act at benzodiazepine binding sites or related sites, and of drugs that can reverse these effects

Drug	Geller-Seifter		Vogel		Social interaction		Drug discrimination (Generalisation to PTZ)		Others
	Effect	Reversal	Effect	Reversal	Effect	Reversal	Effect	Reversal	
Ro 15-1788	No effect 20 mg/kg (Prado de Carvalho et al. 1983)	–	No effect (Corda et al. 1983) 2 mg/kg	–	10 mg/kg (File et al. 1982a)	CDP (5 mg/kg for 5 days) (File and Pellow 1984a); β-CCE (1 mg/kg) (File et al. 1982a); No effect CGS 8216 (File and Lister 1983b)	No effect up to 40 mg/kg (Gherezghiher and Lal 1982; Kehr and Stephens 1984)	–	Increased response to novelty in open field 8 mg/kg (Hoffman and Britton 1983)
CGS 8216	–	–	5–10 mg/kg (Mendelson et al. 1983); 2 mg/kg (Petersen et al. 1983); No effect 1 mg/kg (Corda et al. 1983)	Pentobarbital 5 mg/kg (Mendelson et al. 1983)	10 mg/kg (File and Lister 1983a)	No effect Ro 15-1788 β-CCE (File and Lister 1983b) or CDP (File and Pellow 1984a)	ED_{50} 10 mg/kg (Kehr and Stephens 1984)	–	–
β-CCE	–	–	0.2–0.4 mg/kg (Corda et al. 1983); 10 mg/kg (Petersen et al. 1983)	–	1–2 mg/kg (File et al. 1982a)	Ro 15-1788 10 mg/kg (File et al. 1982a); No effect CGS 8216 (File and Lister 1983b)	–	–	Acute behavioural anxiety syndrome in rhesus monkey 2.5 mg/kg (Ninan et al. 1983) reversed by Ro 15-1788 5 mg/kg
FG 7142	(Rossier et al. 1983)	–	IC30 1.8 mg/kg (Corda et al. 1983); 10 mg/kg (Petersen et al. 1983)	Ro 15-1788 2 mg/kg; CGS 8216 1 mg/kg; Diazepam 0.5 mg/kg (Corda et al. 1983)	5–20 mg/kg (File et al. 1984c)	CDP (5 mg/kg); Ro 15-1788 (10 mg/kg) Not CGS 8216 (10 mg/kg) (File and Pellow 1984c)	ED_{50} 2 mg/kg (Kehr and Stephens 1984)	Ro 15-1788 40 mg/kg (Kehr and Stephens 1984)	Anxiogenic in man (Dorow et al. 1983); Behavioural "anxiety" syndrome in cats (10 mg/kg) reversed by Ro 15-1788 (10 mg/kg); (Ongini et al. 1983)
β-CCM	1 mg/kg (Prado de Carvalho et al. 1983)	–	IC_{30} 0.1 mg/kg (Corda et al. 1983)	Ro 15-1788 2 mg/kg; CGS 8216 1 mg/kg; Diazepam 0.5 mg/kg (Corda et al. 1983)	–	–	(Lal et al. 1982)	–	–

DMCM	–	–	0.2 mg/kg (Corda et al. 1983); 0.5 mg/kg (Petersen et al. 1983)	Ro 15-1788 2 mg/kg; CGS 8216 1 mg/kg; Diazepam 0.5 mg/kg (Corda et al. 1983)	–	–	ED_{50} 0.3 mg/kg (Kehr and Stephens 1984)	Ro 15-1788 40 mg/kg (Kehr and Stephens 1984)	–
β-CCP	–	–	–	–	2 mg/kg (File et al. 1984a)	–	–	–	–
Ro 5-4864	–	–	–	–	5–20 mg/kg (File and Lister 1983c)	Phenytoin 10 mg/kg (File and Lister 1983c); CDP (5 mg/kg for 5 days) (File and Pellow 1984b) Not CGS 8216, Ro 15-1788 (File and Pellow 1984b) or PK 11195 (File and Lister 1983)	–	–	–
Picrotoxin	0.75 mg/kg (Prado de Carvalho et al. 1983b)	–	–	–	2–4 mg/kg (File and Lister 1984)	CDP 5 mg/kg (File and Lister, 1984)	Partial generalisation (Shearman and Lal 1980)	–	–
Pentylene-tetrazol	15 mg/kg (Prado de Carvalho et al. 1983b)	–	15 mg/kg (Corda et al. 1983)	Not by Ro 15-1788 (Corda et al. 1983)	5–20 mg/kg (File and Lister 1984)	CDP 5 mg/kg (File and Lister 1984) not by Ro 15-1788 10 mg/kg (File and Pellow in preparation)	Used as anxiogenic IDS (see Lal and Shearman 1983)	Benzodiazepines (Shearman and Lal 1980; Shearman et al. 1979)	Anxiogenic in man (Rodin 1958; Rodin and Calhoun 1970)
Ro 5-3663	–	–	–	–	2–4 mg/kg (File and Pellow 1983b)	CDP 5 mg/kg (File and Pellow 1983b)	–	–	–

benzodiazepines induce a different conformational change from β-CCE, CGS 8216 and Ro 15-1788. However, the functional relevance of photoaffinity labelling is unclear: Brown and Martin (1983) have recently shown that it may depend more on the chemical structure of the ligand than its pharmacological efficacy.

If the multiplicity of benzodiazepine receptors is at all relevant for the mediation of anxiogenic effects, then it is unlikely to be in a simple manner, e.g. one site for anxiolytic actions and another for anxiogenic actions, particularly since Ro 15-1788 and CGS 8216 show a homogenous binding profile like benzodiazepines. However, some of our behavioural data suggest that at least Ro 15-1788 and FG 7142 are not acting via identical sites on this complex to produce their anxiogenic effects. The ability of chlordiazepoxide to reverse the anxiogenic actions in the social interaction test of compounds acting at this complex differs considerably. As previously described, in the case of FG 7142, acute treatment is sufficient to reverse its effect (File and Pellow 1984c). However, in the case of Ro 15-1788, chronic treatment with chlordiazepoxide is necessary before a reversal can be obtained (File and Pellow 1984a). Chronic treatment with chlordiazepoxide enhances its anxiolytic efficacy in various animal tests (File 1980; Soubrié et al. 1972; Margules and Stein 1968) and clinically (Rickels et al. 1977; Warner 1965). It is often more difficult for a pharmacological reversal to be observed with an antagonist that acts at the same site as the agonist, but with non-competitive antagonism reversal may more easily be seen because both drugs are acting via different receptor populations to produce their effects. From this it follows that chlordiazepoxide is probably acting at the same site as Ro 15-1788 to reverse its anxiogenic action. Acute chlordiazepoxide significantly reversed the anxiety produced by FG 7142, which supports the biochemical evidence reviewed above that the mechanism of action for β-carbolines may be slightly different from that of benzodiazepines (and Ro 15-1788). This idea is supported by evidence (reviewed in section 3) that chlordiazepoxide is able to completely reverse the anxiogenic actions of drugs thought to act via the picrotoxin site after acute treatment. Other behavioural evidence also suggests that FG 7142 has a different mechanism of action from Ro 15-1788: in contrast to Ro 15-1788, FG 7142 reversed the anticonflict effect of phenobarbital as well as that of lorazepam in rats (Petersen et al. 1982).

Ehlert et al. (1983) believe that it is difficult to rationalise the concept of distinct β-carboline sites. For example, the negative cooperativity between the two sites would have to be great so that the interaction between these ligands would appear competitive. Instead, Ehlert et al. (1983) suggest a different model to account for the behavioural interactions of these drugs. It has been proposed (Ehlert et al. 1981; Braestrup and Nielsen 1981) that the ability of GABA agonists to reduce or enhance the affinity of ligands for the benzodiazepine receptors reflects the pharmacological efficacy of benzodiazepine receptor ligands, and that they may reflect the ability of these ligands to enhance or reduce GABA-mediated chloride channel conductance. Benzodiazepine receptor ligands can be roughly divided into three overlapping groups according to whether muscimol enhances, reduces or leaves unaffected their affinity (Braestrup et al. 1982). Group 1 represents ligands with benzodiazepine-like activity, and muscimol enhances their affinity. Group 2 represents ligands, e.g. Ro 15-1788 and β-CCP which are unaffected by muscimol and therefore thought to have little or no efficacy at benzodiazepine receptors (i.e. they bind to the receptors but induce no conformational change, and therefore have no intrinsic actions). Group 3 represents ligands, e.g. FG 7142, β-CCE, β-CCM and DMCM which are anxiogenic and proconvulsant or convulsant, and muscimol decreases their affinity. CGS 8216 also belongs in this group (Morelli et al. 1982). However, Braestrup et al. (1983) show that the GABA ratio is not fully predictive of pharmacological efficacy: the β-carboline receptor antagonist ZK 93426 has a GABA ratio like that of benzodiazepines.

Braestrup et al. (1983) have also suggested that chloride channel-related interactions could identify pharmacological efficacy, i.e. whether chloride ions and barbiturates enhance, decrease or leave unaffected the binding of drugs to the benzodiazepine site. Chloride ions increase the binding of both benzodiazepines and DMCM and so this is clearly not a good predictor of efficacy. Barbiturates enhance the binding of benzodiazepines but decrease the binding of DMCM and β-CCM, which suggests that the barbiturate shift may be a better predictor. However, this system is also far from foolproof: Braestrup et al. also show that two benzodiazepine receptor agonists, ZK 93423 and CL 218,872, and the partial agonist ZK 91296 do not have the barbiturate shifts that would be expected from their pharmacological actions.

From the general pattern described above, Braestrup et al. (1982, 1983) proposed that there are two conformations of benzodiazepine sites which are in equilibrium. Opening of neuronal chloride channels by GABA is dependent on an activated conformation ("open chloride channel" form). Benzodiazepines shift the equilibrium to stabilise this conformation. β-carbolines, which may have high affinity for the closed chloride channel conformation, would reduce the probability of opening chloride channels and thus reduce GABA-mediated neurotransmission. Ro 15-1788 and β-CCP do not favour either conformation and therefore do not affect GABA-mediated neurotransmission (and so have no intrinsic pharmacological actions). However, these drugs can obstruct other ligands and therefore antagonise agents acting on both conformations. A basic problem with this simple model is that it does not account for the anxiogenic actions of Ro 15-1788 and of β-CCP. In fact, it is possible that Ro 15-1788 and β-CCP belong in the same category as CGS 8216 and other β-carbolines, as the evidence for the effect of GABA on these latter compounds is equivocal: there is evidence that GABA either decreases or leaves unaffected the affinity of drugs in Braestrup's Group 3. Recently, Chiu and Rosenberg (1983) have presented evidence, based on its binding kinetics, that Ro 15-1788 is a powerful ligand in inducing conformational changes in the benzodiazepine binding complex that may be functionally relevant. This mechanism is not inconsistent with the increasing accumulation of data showing that, contrary to initial reports, Ro 15-1788 has clear intrinsic behavioural actions, as well as the ability to antagonise the actions of both anxiolytic and other anxiogenic ligands of benzodiazepine binding sites.

However, the picture with CGS 8216 is quite different. The anxiogenic actions of this compound are not blocked by Ro 15-1788, by β-CCE, by FG 7142 or by chlordiazepoxide (File and Lister 1983b, File and Pellow 1984c; File and Pellow in preparation). This strongly suggests

that these actions of CGS 8216 may not be mediated via the classical CNS benzodiazepine binding sites. In this respect it is worth noting that the doses at which intrinsic actions of CGS 8216 are seen (5 – 10 mg/kg) are approximately ten times higher than those reported to antagonise the effects of benzodiazepines. CGS 8216 has no action at peripheral-type benzodiazepine binding sites (Czernik et al. 1982; Schoemaker et al. 1983): however, its actions at the picrotoxinin site have not yet been reported. CGS 8216 inhibits adenosine activation of cyclic AMP formation (Czernik et al. 1982) and so an action via adenosine sites is one possibility.

c) Electrophysiology

Polc et al (1982) showed that β-CCE and β-CCM depressed segmental dorsal root potentials in spinal cats and reversed the prolongation of dorsal root potentials by phenobarbitone. β-CCE also enhanced the low-frequency facilitation of pyramidal population spikes in the hippocampus of anaesthetised rats. Ro 15-1788, while affecting none of these measures alone, abolished the actions of β-CCE and β-CCM. Nutt et al. (1982) showed that Ro 15-1788 blocked the effects of β-CCE on GABA-induced depolarisation in isolated cervical sympathetic ganglia. Ro 15-1788 also antagonises the electrophysiological actions of benzodiazepines (Nutt et al. 1982; Polc et al. 1981). On the basis of these results Polc et al. (1982) proposed a three-state model of the benzodiazepine receptor that follows essentially the same principles as that of Braestrup et al. (1982, 1983, see above), and has the same major limitation: it cannot explain the intrinsic behavioural actions of Ro 15-1788. Recent work has shown that Ro 15-1788 does have intrinsic actions in electrophysiological preparations: opposite to benzodiazepines, at 0.1 – 1.0 μM it decreases several indices of inhibition in the CA1 region of the in vitro hippocampal slice preparation (King et al. 1983).

3. Drugs acting at the picrotoxinin site

a) Behaviour

Picrotoxin is a convulsant agent for which there are specific binding sites in the CNS related to the chloride channel, forming part of the GABA receptor-benzodiazepine binding site – chloride ionophore complex (see Table 1). *Pentylenetetrazol,* another convulsant drug, is also thought to act at this site (Olsen and Leeb-Lundberg 1981; Simmonds 1982). *Ro 5-3663,* in contrast to other 1,4-benzodiazepines, has a behavioural and biochemical profile similar to that of picrotoxin. It is inactive at the classical CNS benzodiazepine binding site (Leeb-Lundberg et al. 1981) and at peripheral-type benzodiazepine sites (Schoemaker et al. 1981) but displaces [^{3}H]-α-dihydropicrotoxinin from its binding sites (Leeb-Lundberg et al. 1981). Ro 5-3663 is a convulsant which shows neuronal excitatory activity in the CNS and has GABA antagonist activity (Schlosser and Franco 1979; Harrison and Simmonds 1983).

Early reports suggested that pentylenetetrazol caused intense anxiety in patients (Rodin 1958; Rodin and Calhoun 1970). As described in section 1, Lal and Shearman (1982) have shown that rats can learn to discriminate between saline and PTZ, and they believe that the IDS produced by PTZ is related to its anxiongenic action. Interestingly, the discrimination only partially generalised to picrotoxin, and higher doses could not be investigated because they entered the convulsant range (Shearman and Lal 1980).

Subconvulsant doses of picrotoxin, PTZ and Ro 5-3663 were examined in the social interaction test. Picrotoxin (2 and 4 mg/kg) and Ro 5-3663 (2 and 4 mg/kg) produced significant reductions in time spent in active social interaction, with reductions in locomotor activity seen only at the higher dose (File and Lister 1984; File and Pellow 1983b). PTZ (5, 10 and 20 mg/kg) also produced a dose-related reduction in social interaction, but at the dose at which significance was reached, there was also a reduction in locomotor activity (File and Lister 1984). Although it seems likely that this is merely secondary to the reduction in social interaction, an anxiogenic interpretation here is less clear. PTZ (15 mg/kg) reduces the number of shocks taken by rats in a punished drinking test (Corda et al. 1983), and both picrotoxin (0.75 mg/kg) and PTZ (25 mg/kg) decreased punished but not unpunished responding in a modified Geller-Seifter conflict procedure in mice (Prado de Carvalho et al. 1983b).

The reduction in social interaction elicited by each of these drugs was reversed by chlordiazepoxide (5 mg/kg, File and Lister 1984; File and Pellow 1983b). However, the anxiogenic actions of picrotoxin and PTZ in the social interaction test were not reversed by Ro 15-1788 (10 mg/kg, File and Pellow in preparation). Likewise, Ro 15-1788 did not reverse the anxiogenic action of PTZ in a punished drinking test (Corda et al. 1983).

b) Biochemistry

The anxiogenic action of picrotoxin, pentylenetetrazol and Ro 5-3663 suggests that this site also may be able to mediate anxiogenic effects. The reversal of these effects by chlordiazepoxide is not inconsistent with such a suggestion, as chlordiazepoxide does displace [^{3}H]-α-dihydropicrotoxinin from its binding sites, albeit in the micromolar range (Leeb-Lundberg et al. 1981). However, given that the picrotoxinin site and the classical CNS benzodiazepine binding site coexist on a supramolecular complex in the CNS, it is more likely that chlordiazepoxide may block the anxiogenic action of these drugs via an allosteric interaction with picrotoxinin binding sites. That chlordiazepoxide is able to reverse these effects after acute treatment is consistent with such a suggestion (see section 2b). The biochemistry of the picrotoxinin site has been comparatively neglected, and the actions of anxiogenic drugs other than picrotoxin, PTZ and Ro 5-3663 at these sites have not yet been reported. Until recently, however, binding to the picrotoxinin site has been rather difficult technically, because picrotoxin has only very low affinity for these sites, and a large proportion of picrotoxin binding in the CNS is non-specific (85 – 90%). However, with the discovery of a new high affinity ligand for these sites (TBPT, K_d 25 nM, Ramanjaneyulu and Ticku 1983) it is possible that investigations into the biochemistry of this site will increase.

4. Ro 5-4864

Ro 5-4864 is an atypical 1,4-benzodiazepine that differs structurally from diazepam only by a *p*-chloro substituent. Because its sites of action have not been clearly delineated,

it will be considered separately until a clear classification becomes possible. Ro 5-4864 has very low affinity for the classical CNS benzodiazepine binding sites, and yet high affinity for peripheral-type sites (see Table 1). Although initially this compound was found to produce tranquilisation and drowsiness in man (Zbinden and Randall 1967), it has long been classified as inactive (e.g. Richards et al. 1982). However, clear intrinsic actions have recently been identified for this drug (see Pellow and File 1984 for review). At doses below those that produce sedation (File and Pellow 1983a) and convulsions (File and Mabbutt 1983; File et al. 1984b; Pieri et al. 1983; Weissman et al. 1983), Ro 5-4864 (5–20 mg/kg) produces a significant reduction in active social interaction, with a decrease in locomotor activity only at the 20 mg/kg dose (File and Lister 1983d). *PK 11195* is an isoquinoline carboxamide derivative with a high affinity for [^{3}H]-Ro 5-4864 recognition sites in the periphery and the brain (LeFur et al. 1983a, b) and is thought on the basis of controversial thermodynamic data to be an antagonist there (LeFur et al. 1983c). This drug (10 mg/kg) had no effect on the anxiogenic action of Ro 5-4864 (File and Lister 1983d), implying that the anxiogenic action of Ro 5-4864 may not be mediated via peripheral-type sites.

Other data support this suggestion. Chlordiazepoxide is a benzodiazepine that is inactive at these sites (Schoemaker et al. 1981: less than 30% inhibition at 10 μM), and yet chlordiazepoxide (5 mg/kg) given chronically for 5 days prior to testing, significantly reversed the anxiogenic action of Ro 5-4864 (File and Pellow 1984b). It is unlikely that the anxiogenic action of Ro 5-4864 is mediated via benzodiazepine binding sites, since Ro 15-1788 (10 mg/kg), which is inactive at peripheral-type sites (Richards et al. 1982; Schoemaker et al. 1983: $K_i > 10$ μM), failed to reverse it (File and Pellow 1984b). The anticonvulsant *phenytoin*, in addition to other actions, displaced [^{3}H]-diazepam from "micromolar" binding sites in the CNS (Bowling and DeLorenzo 1982a). Phenytoin (10 mg/kg) reversed the anxiogenic action of Ro 5-4864 (File and Lister 1983d), suggesting a role for low affinity binding sites in mediation of this anxiety.

Although the existence of micromolar benzodiazepine sites linked to the calcium-calmodulin kinase system has not been confirmed (e.g. File et al. 1984b), low affinity binding sites for benzodiazepines are a possibility that cannot be ignored. Most receptor binding studies, for technical reasons, are limited in the range of ligand concentrations tested; consequently such low affinity states for benzodiazepine binding would not be measured. One low affinity site that could be mediating some actions of benzodiazepines is the specific phenytoin binding site in the CNS (Shah et al. 1981; Burnham et al. 1981; Bowling and DeLorenzo 1982b). However, there is some controversy over whether benzodiazepines enhance (Shah et al. 1981; Okazaki et al. 1983) or inhibit (Bowling and DeLorenzo 1982b) the binding of [^{3}H]-phenytoin to these sites.

Another site that could mediate the anxiogenic action of Ro 5-4864 is the picrotoxin site. Initial studies using [^{3}H]-α-dihydropicrotoxinin as a ligand suggested that Ro 5-4864 has a very low affinity for this site (Leeb-Lundberg et al. 1981). Recent biochemical studies, however, are providing evidence that Ro 5-4864 may have some action via the GABA-benzodiazepine-ionophore complex in the CNS. McNeil et al. (1983) found that 1 μM Ro 5-4864 inhibits GABA enhancement of [^{3}H]-diazepam binding to rat cortex, and blocked GABA-mediated excitatory effects of flunitrazepam on dopamine neurone firing rates. Also, Squires and Saederup (1983) found that Ro 5-4864 (1 μM) partially reversed the inhibitory effect of GABA (5 μM) on TBPT binding to the picrotoxinin site. This ligand has properties similar, but not identical, to dihydropicrotoxinin. Ticku and Ramanjaneyulu (1984) also investigated the effect of Ro 5-4864 on TBPT binding to picrotoxinin binding sites, and found that it inhibits TBPT binding, apparently competitively, with an IC_{50} value of approximately 20 μM. At present there is very little biochemical data available on the mechanisms of action of Ro 5-4864 and there has been no detailed investigation into the biochemistry of peripheral-type binding sites as there has been for classical CNS sites.

Recent electrophysiologic also suggests that Ro 5-4864 may have some action at the GABA-benzodiazepine-ionophore complex. Polc and Schaffner (1983), in cats, showed that Ro 5-4864 depressed dorsal root reflexes, and presynaptic inhibition as well as the early part of segmental dorsal root potentials. Spontaneous and evoked activity of γ-motoneurones and Renshaw cells was increased. There was no alteration of the population spike size of CA1 pyramidal cells, but reduced recurrent inhibition, and dose-dependent enhancement of low frequency facilitation of these neurones. These effects of Ro 5-4864 in the spinal cord and hippocampus are partially antagonised by Ro 15-1788. Clearly, this work correlates with behavioural evidence for a reversal by Ro 15-1788 of the convulsions and the reduction in rearing in the holeboard caused by Ro 5-4864 (File et al. 1984b; File and Pellow 1983a), although the mechanism by which Ro 15-1788 is able to do this remains unclear. Pellow et al. (1984b) report that, in an in vitro preparation of rat cuneate nucleus, low concentrations of Ro 5-4864 (0.1 μM) antagonised the effects of flurazepam (1 μM) as a potentiator of muscimol. Higher concentrations (30 μM) had three additional effects: antagonism of the effect of pentobarbitone (10 μM) as a potentiator of muscimol; enhancement of the effects of picrotoxin (10 μM) as an antagonist of muscimol, and a small direct antagonism of muscimol.

Behavioural, electrophysiological and biochemical evidence suggest that the actions of this drug cannot by explained only by its interactions with peripheral-type benzodiazepine binding sites. Although at present there is insufficient evidence to clearly identify an alternative site of action for Ro 5-4864, some action via the picrotoxin site seems to be the most likely possibility in the light of recent evidence.

5. Conclusion

In conclusion, there are now several behavioural tests that are sufficiently sensitive to identify both anxiolytic and anxiogenic drug profiles. Although there are problems with each of these tests individually, in general there is a good consensus on identification of anxiogenic compounds. It is clear from the three types of evidence discussed above that at least two sites on the GABA-benzodiazepine-ionophore complex can mediate anxiogenic effects: the benzodiazepine receptor (and a distinct β-carboline site?) and the picrotoxin site. An interesting question that cannot be answered at present is whether the nature of the anxiety produced via

the picrotoxin site is different from that produced via the benzodiazepine/β-carboline sites.

As one would expect, indices of drug function provided by behavioural and electrophysiological data are highly correlated: drugs that have intrinsic actions in behavioural tests also have intrinsic actions in electrophysiological preparations, and the patterns of reversal by other drugs are very similar. However, it is clear from preceding sections that the classification of these compounds according to data from ligand binding studies does not correlate highly with their pharmacological interactions in behavioural and electrophysiological procedures. It must be stressed that the latter procedures are measuring functional systems in vivo and in vitro, whereas biochemical techniques that simply identify the sites to which drugs bind produce no information about functional consequences of the drugs. In fact, it is not clear at present to what extent it is meaningful to correlate the functional consequences caused by a drug with its site of action, and before any firm conclusions can be drawn from binding studies, further biochemical evidence is necessary to provide some measure of the drug's efficacy at these sites once it is bound there.

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Received January 24, 1984; Final version March 12, 1984