Br. J. Pharmac. (1986), **88**, 507–514

The effects of drugs acting at the $GABA_A$-receptor/ionophore after chemical kindling with the benzodiazepine receptor ligand FG 7142

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1 Repeated administration of the β-carboline benzodiazepine receptor ligand FG 7142 produces sensitization to its effects so that full seizures develop (chemical kindling); initially it is only proconvulsant. The present study investigated alterations in the function of drugs which act at the different sites at the γ-aminobutyric acid (GABA) benzodiazepine receptor complex, after repeated administration of FG 7142.

2 In FG 7142 kindled mice decreased anticonvulsant and hypothermic effects of the GABA agonist muscimol were observed. The hypothermic effects of the GABA agonist progabide were reduced. In contrast a small increase in the hypothermic effect of pentobarbitone was seen.

3 The convulsant effects of bicuculline and picrotoxin were unaltered when they were given intravenously but marginally increased when they were given by the intraperitoneal route. No changes were seen in the hypothermic effects of these drugs.

4 No significant changes were seen in the convulsant or hypothermic effects of pentylenetetrazol.

5 These results suggest that kindling with FG 7142 may alter GABA receptor function.

Introduction

The GABA-receptor/ionophore complex is a protein which includes binding sites for γ-aminobutyric acid (GABA),benzodiazepines, picrotoxin and barbiturates, and the chloride channel. Compounds which bind to benzodiazepine receptors can either have actions like those of the benzodiazepines, anxiolytic and anticonvulsant (agonists), or the opposite effects, causing anxiety and convulsions (inverse agonists or contragonists) (Oakley & Jones, 1980; Cowen *et al.*, 1981; Dorow *et al.*, 1983). The latter group include β-carboline compounds such as β-CCE (ethyl-β-carboline-3-carboxylate) and FG 7142 (N-methyl-β-carboline-3-carboxamide) and the effects of these, as well as those of benzodiazepines, are blocked by the antagonist Ro 15-1788 (Hunkeler *et al.*, 1981; Nutt *et al.*, 1982). These effects of these three types of compound are reflected in their effects on the actions on the inhibitory transmitter GABA (Little, 1984), and this is thought to be the basis of their *in vivo* actions.

We have recently shown that treatment of mice for twelve days with FG 7142 leads to a sensitization to its effects, resulting in a change from pro-convulsant to full convulsive effects (Little *et al.*, 1984). This type of increase in the effects of excitatory drugs is known as chemical kindling, by analogy with electrical kindling (Goddard, 1967; Racine, 1978). We now present the results of studies designed to determine whether or not the treatment with FG 7142 causes changes in the effects of drugs acting at sites other than the benzodiazepine receptor, on the GABA-receptor/ionophore complex. The drugs used in the challenge tests in this study were muscimol, progabide, bicuculline, picrotoxin, pentobarbitone and pentylenetetrazol (PTZ). The competitive antagonist of GABA, bicuculline, acts directly at the GABA receptor, as do the agonists used, muscimol and progabide (Bartholini *et al.*, 1979). Picrotoxin is thought to bind to a site adjacent to the GABA and benzodiazepine receptors and cause convulsions by decreasing the effects of GABA on the chloride channel (Olsen, 1981; Simmonds, 1980). Pentobarbitone potentiates the effects of GABA (Barker *et al.*, 1981; Harrison & Simmonds, 1983). It is thought to bind to a site closely related to but distinct from the picrotoxin site (Ticku & Olsen, 1978; Macksay & Ticku, 1985). although it does have other neurophysiological actions (Goldring & Blaustein, 1980). We also studied the effects of PTZ, a

convulsant which has been shown to bind to the picrotoxin receptor site (Ramanjaneyulu & Ticku, 1984).

There have been few studies on the mechanism of chemical kindling, although electrical kindling has been more fully investigated and is thought not to be due to changes in any single transmitter system. Administration of benzodiazepines or GABA transaminase inhibitors slowed electrical kindling but this may simply reflect their anticonvulsant properties rather than any involvement in the mechanism of kindling (Albersen *et al.*, 1980; Le Gal La Salle, 1980; Kalishman *et al.*, 1982). Alterations in GABA transmission after electrical kindling have been suggested by studies on GABA release and on muscimol binding (Liebowitz *et al.*, 1977), but evidence for direct involvement is lacking. Recent studies have suggested that changes in somatostatin concentrations may underlie the kindling phenomenon (Higuchi *et al.*, 1983, 1984; Assouline *et al.*, 1984). Somatostatin has been shown to co-exist with GABA in the dorsolateral geniculate nucleus (Sillito *et al.*, 1984).

A preliminary account of some of these results was presented to the British Pharmacological Society, December 1984.

Methods

In these studies we have used simple behavioural and physiological tests to determine whether or not chronic treatment with FG 7142 alters the effects of drugs acting at sites on the GABA-receptor/ionophore. In each case we used two or more methods in order to determine the activity of a drug. The chronic treatment with FG 7142 (see below) was kept constant, using a 12 day schedule which we have shown previously to be optimal in this strain of mice (Little *et al.*, 1984). In all cases the 'challenge' tests on the acute actions of the drugs under study were carried out one week after the end of the twelve day treatment (day 19), a time at which we know the kindling phenomenon is still present. In order to avoid chronic treatment of very large numbers of animals the dose-response curves of the drugs used for the acute studies were carried out for each test in naive animals and doses in the middle of each dose range chosen for the challenge tests. In every challenge test the experimenter did not know the prior treatment.

Statistical analyses were made using Fisher's exact test for the convulsion incidences and the Mann Whitney U test for the rest of the data. In the

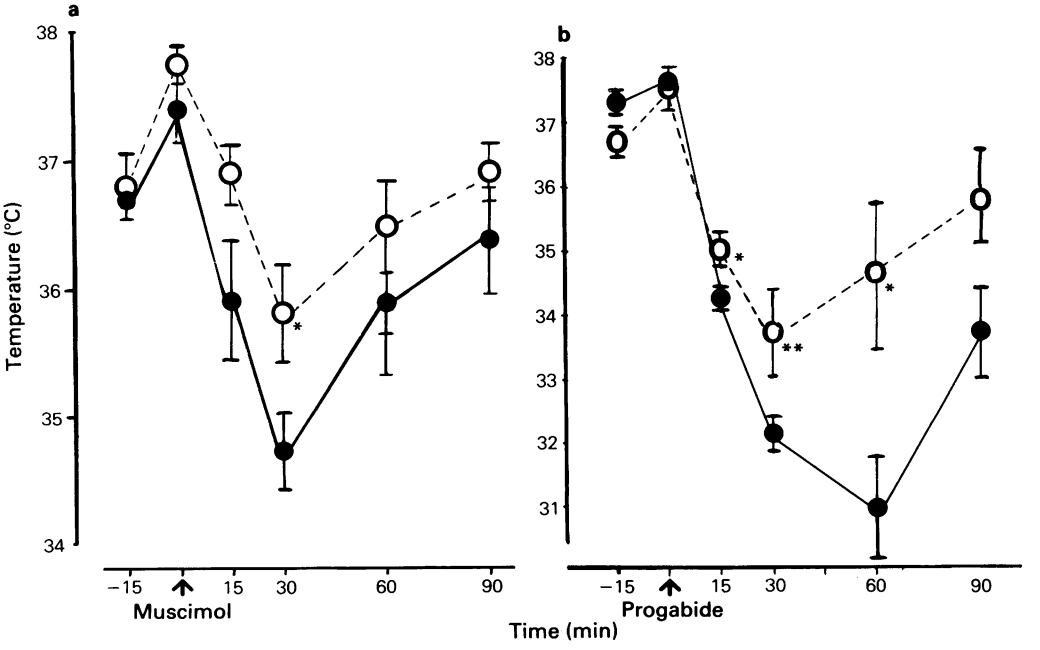

Figure 1 The hypothermic effects of (a) muscimol and (b) progabide in control (injected with Tween vehicle, ●) and FG 7142 kindled (○) mice. Body temperatures were measured at the times shown, before and after intraperitoneal injection of (a) muscimol 1 mg kg^{-1} and (b) progabide 200 mg kg^{-1}. Points are mean, with vertical lines indicating s.e.mean, $n = 8$. $^{*}P < 0.05$, $^{**}P < 0.02$.

temperature measurements comparisons were made between measurements at each time interval after injection.

Chronic treatment

Male CD1 mice (30–35 g) were used throughout. FG 7142, 40 mg kg^{-1}, 10 ml kg^{-1}, was injected i.p. once daily for twelve days. (This dose was found acutely to have the maximal proconvulsant effect (Little *et al.*, 1984). On days 10, 11 and 12 (day 1 = first injection) the mice, marked individually, were observed for 1 h after injection and the incidence of full convulsions noted. A full convulsion was defined as contractions of the muscles of limbs and body accompanied by loss of posture. Groups of mice in which at least 6/8 animals convulsed during these three days were used for the challenge studies. Control animals received vehicle injections (Tween 80, one drop in 10 ml distilled water).

Acute 'challenge' studies

(i) *Convulsion incidence* Groups of mice (eight at a time, marked individually) were watched for 1 h after injection and the numbers of full convulsions counted (see above for definition of full convulsion). Myoclonic jerks and other behaviours were also noted.

(ii) *Convulsion thresholds, infusion method* Thresholds for convulsions to PTZ and bicuculline were determined by intravenous infusion (Nutt *et al.*, 1980). A 'butterfly' cannula was inserted into the tail vein and the convulsant infused until the first clear signs of clonic convulsions were seen, i.e. contractions of limb muscles. The animals were killed as soon as the first signs of convulsive activity were seen. The thresholds were determined from the amount of convulsant needed to cause a convulsion, derived from the volume of infusion needed. The infusion rates used are given in the appropriate Results sections. Anticonvulsant effects of the drugs were measured by their actions against intravenous infusions of PTZ. No significant differences were found in the baseline thresholds to PTZ but, to ensure that this remained the case, the baseline thresholds were repeated in every experiment in which anticonvulsant effects of drugs were studied.

(iii) *Temperature measurements* Temperatures were measured by a rectal probe inserted 2 cm, connected to a thermometer. As the body temperatures of mice are very labile an experimental schedule was used which was designed to demonstrate most clearly the effects of the drugs. For every experiment the controls and test animals were measured concurrently. The two groups were individually marked and kept in the same cage overnight. This minimized differences such as those due to fighting in the cages before the experiment. All measurements were started at 10 h 00 min, the animals being disturbed as little as possible before the start of the experiment. Mice from each group were taken alternately out of the cage and their temperatures measured. They were then weighed and given any further coding necessary, and returned to the home cage. Fifteen minutes later the temperatures were measured again in the same order as before, and the injections of the drug to be studied that day or the vehicle, were given. Temperature measurements were then made 15, 30, 60 and 90 min later. This procedure ensured that the temperature rise caused by the first handling of the animals did not mask the effects of the drugs to be studied, and also provided measurement of temperatures before the acute drug administration. Ambient temperature was kept at 22°C. In the absence of drug treatment the temperatures of control mice gradually decline from the rise due to handling plus vehicle injections to the original temperatures, over about 90 min (Taylor *et al.*, 1985).

Table 1 Convulsion thresholds to pentylenetetrazol (PTZ) 30 min following the injection of the drugs shown, after kindling with FG 7142

Chronic treatment	*Challenge day 19*	*PTZ* (mg kg^{-1} i.v.)
Tween	Saline	39 ± 2 (7)
FG 7142	Saline	37 ± 1 (6)
Tween	Muscimol, 1 mg kg^{-1}	49 ± 3 (8)
FG 7142	Muscimol, 1 mg kg^{-1}	38 ± 3 (8)**
Tween	Muscimol, 2 mg kg^{-1}	47 ± 5 (7)
FG 7142	Muscimol, 2 mg kg^{-1}	51 ± 4 (6)
Tween	Muscimol, 3.5 mg kg^{-1}	50 ± 4 (5)
FG 7142	Muscimol, 3.5 mg kg^{-1}	53 ± 3 (7)
Tween	Saline	27 ± 2 (7)
FG 7142	Saline	30 ± 2 (8)
Tween	Progabide,	30 ± 2 (4)
FG 7142	100 mg kg^{-1}	34 ± 4 (8)
Tween	Progabide,	55 ± 5 (4)
FG 7142	200 mg kg^{-1}	63 ± 5 (8)
Tween	Progabide,	77 ± 5 (7)
FG 7142	400 mg kg^{-1}	77 ± 8 (6)
Tween	Saline	37 ± 1 (8)
FG 7142	Saline	35 ± 3 (8)
Tween	Pentobarbitone,	65 ± 7 (7)
FG 7142	20 mg kg^{-1}	70 ± 5 (8)

The infusion concentration was 2.5 mg ml^{-1} and infusion rate 1.1 ml min^{-1}. Data shown are means ± s.e.mean of number in parentheses. **$P < 0.02$

Drugs

FG 7142 (N-methyl-β-carboline-3-carboxamide, A/S Ferrosan, Denmark) and progabide (Synthelabo, France) were suspended in distilled water with 1 drop of Tween-80 per 10 ml; muscimol, PTZ and picrotoxin (Sigma) were dissolved in saline; bicuculline (Sigma) was dissolved in 0.1 N HCl, titrated to pH 3 with NaOH, then diluted with saline brought to pH 3 with HCl.

Results

(i) *The effects of muscimol and progabide*

The hypothermic effects of muscimol were decreased in kindled animals compared with controls (Figure 1a); the difference was significant at 30 min after administration ($P < 0.05$). The hypothermic effect of 0.5 mg kg^{-1} muscimol was also decreased in kindled animals (not illustrated), and this was significant at the 15 min time interval. When the anticonvulsant effects of muscimol against PTZ infusion were studied it was significantly ($P < 0.02$) less effective in kindled animals when given at a dose of 1 mg kg^{-1} (Table 1). No differences were seen at higher doses but at these the anticonvulsant effects of muscimol appeared to reach a plateau so it is unlikely that changes would have been seen. Muscimol causes convulsions at higher doses (4 mg kg^{-1} and above) in this strain of mice; this may have limited its anticonvulsant action. Progabide was also less hypothermic in kindled animals (Figure 1b), the difference being significant at 15, 30 and 60 min after injection. However, there were no significant differences in its anticonvulsant action against PTZ (Table 1).

(ii) *The effects of bicuculline*

The effects of bicuculline after FG 7142 kindling are shown in Figure 2a and Table 2. No significant differences were seen in the hypothermic actions or the intravenous infusion thresholds. The effects of an intraperitoneal injection of a submaximally effective convulsant dose (4 mg kg^{-1}) were not significantly different when the numbers of mice convulsing out of each group were compared (Table 2). However, significantly more mice in the FG 7142-treated group had multiple or continuous seizures. In three of the kindled

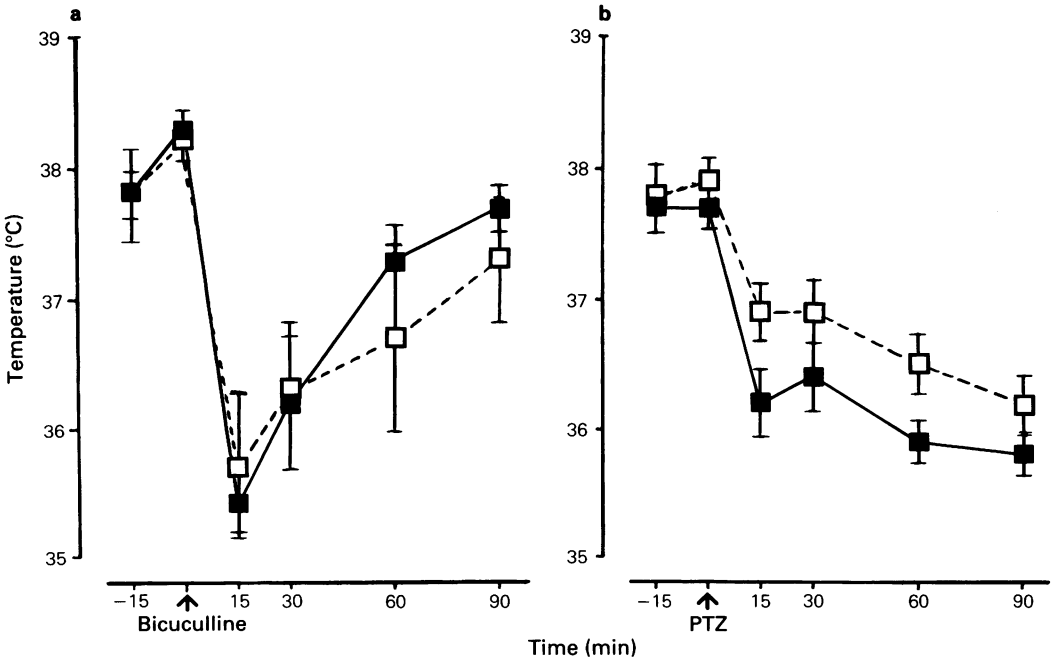

Figure 2 The hypothermic effects of (a) bicuculline (3 mg kg^{-1}) and (b) pentylenetetrazol (PTZ, 20 mg kg^{-1}) in control (injected with Tween vehicle, ■) and FG 7142 kindled (□) mice. Body temperatures were measured at the times shown, before and after intraperitoneal injection of the drugs. Points are mean, with vertical lines indicating s.e.mean, $n = 8$.

Table 2 The convulsive effects of bicuculline, picrotoxin and pentylenetetrazol (PTZ) after kindling with FG 7142

Bicuculline

Convulsion threshold:

Chronic treatment	*Day 19 bicuculline* (mg kg^{-1}, i.v.)
Tween	0.41 ± 0.02 (7)
FG 7142	0.42 ± 0.03 (7)

Convulsion incidence:

Chronic treatment	*Challenge day 19*	*Convulsions*	*Multiple seizures*	*Mortality*
Tween	Bicuculline, 4 mg kg^{-1}	5/7	0/7	0/7
FG 7142		5/7	5/7**	3/7

Picrotoxin

Convulsion incidence:

Chronic treatment	*Challenge day 19*	*Convulsions*	*Multiple seizures*
Tween	Picrotoxin, 2 mg kg^{-1}	0/8	0/8
FG 7142		1/8	0/8
Tween	Picrotoxin, 2 mg kg^{-1}	0/8	0/8
FG 7142		1/9	0/9
Tween	Picrotoxin, 4 mg kg^{-1}	7/7	0/7
FG 7142		7/7	4/7*

PTZ

Convulsion incidence:

Chronic treatment	*Challenge*	*Convulsions*
Tween	PTZ, 40 mg kg^{-1}	3/8
FG 7142		3/7

The rate of infusion of bicuculline was 1.1 ml min^{-1} and the concentration 0.05 mg ml^{-1}. Convulsion incidence refers to the number of animals out of the group showing either full convulsions or multiple seizures (as stated), after the convulsant drugs were given by the intraperitoneal route. *$P < 0.05$, **$P < 0.02$.

mice the bicuculline seizures were fatal, but in none of the vehicle treated mice did this occur; this difference was not significant.

(iii) *The effects of pentylenetetrazol*

No significant differences were found in the intravenous infusion thresholds to PTZ in any experiments. We therefore considered it valid to use PTZ infusion to study the anticonvulsant effects of other drugs. However, we established the thresholds to PTZ in the absence of any other acute treatment in every case and all the values obtained are summarized in Table 3. No significant differences were found in the hypothermic effects of PTZ (Figure 2b) or in the number of animals showing convulsions, after a bolus i.p. injection of 40 mg kg^{-1} (Table 2).

(iv) *The effects of picrotoxin*

After FG 7142 kindling no significant changes were seen in the effects of picrotoxin on body temperature (Figure 3a) or on convulsion incidence (Table 2). The second set of results on convulsion incidence after 2 mg kg^{-1} picrotoxin (Table 2) was obtained during the temperature measurements. The temperature of the one mouse that convulsed followed the same pattern as those of the rest of the group. With the higher dose (4 mg kg^{-1}) multiple seizures were seen in the kindled group but only single convulsions in the controls. There were no deaths. Picrotoxin is not suitable for use in the infusion method for convulsion threshold because there is a latency before the seizures occur.

(v) *The effects of pentobarbitone*

There was no significant difference in the anticonvulsant action of pentobarbitone, 20 mg kg^{-1} against PTZ, after kindling with FG 7142, compared with chronic vehicle-treated controls (Table 1). The hypothermic action of pentobarbitone was marginally

Table 3 Seizure thresholds to pentylenetetrazol (PTZ) following kindling treatment with FG 7142

Chronic treatment	*Day 19, PTZ (i.v.) 5 mg ml^{-1}, 1.1 ml min^{-1}*	
	Myoclonus	*Tonic seizure*
Tween	38 ± 5 (8)	67 ± 3 (8)
FG 7142	40 ± 3 (7)	74 ± 7 (7)
	2.5 mg ml^{-1}, 1.1 ml min^{-1} Myoclonus	
Tween	39 ± 2 (7)	
FG 7142	38 ± 1 (6)	
Tween	38 ± 2 (7)	
FG 7142	38 ± 1 (6)	
Tween	37 ± 1 (8)	
FG 7142	35 ± 3 (8)	
Tween	31 ± 1 (8)	
FG 7142	33 ± 2 (9)	
Tween	27 ± 2 (7)	
FG 7142	30 ± 2 (8)	
	10 mg ml^{-1}, 1.1 ml min^{-1} Myoclonus	
Tween	59 ± 6 (7)	
FG 7142	71 ± 6 (5)	
Tween	47 ± 3 (7)	
FG 7142	48 ± 5 (7)	
Tween	57 ± 4 (9)	
FG 7142	48 ± 3 (9)	

The concentrations of PTZ used were altered as shown. Data shown are means ± s.e.mean of number in parentheses. No significant differences were seen.

greater in kindled animals, the difference being significant ($P < 0.002$) only at 15 min (Figure 3b).

Discussion

The chronic treatment with FG 7142 decreased the effects of the GABA agonists, muscimol and progabide. In most of the tests no changes were seen in the effects of bicuculline or picrotoxin, but there appeared to be a marginal increase in the convulsant effects of these drugs. Little effect was seen on the actions of pentobarbitone; the only significant change was an increased hypothermic action at one time point.

It is clear that kindling to FG 7142 did not involve a general increase in excitability of the CNS, as little change was seen in the responses to bicuculline and picrotoxin and none in responses to PTZ, although the effects of the latter were tested repeatedly.

The changes in the effects of the GABA agonists and antagonists suggest that chronic treatment with FG 7142 decreases GABA receptor function. Such a change could be due to an alteration directly at the GABA-receptor/ionophore or by a change in the coupling between the benzodiazepine receptor site and the GABA-receptor/ionophore. The latter might be caused by modification of the coupling between the two sites or by changes in endogenous ligand action at the benzodiazepine receptor. However, it is not yet known whether or not such a ligand exists (or if it does, whether it has benzodiazepine agonist or inverse agonist properties). Acutely inverse agonist drugs have been shown to reduce the effect of GABA-receptor interactions (Skovgaard *et al.*, 1983; Little, 1984). The findings with kindling suggest that this acute interaction may somehow become persistent and occur despite the removal of the inverse agonist drug.

This suggested alteration is not incompatible with the minimal changes in the effects of bicuculline and picrotoxin, as the effects of these drugs depend on the amount of endogenous GABA activity. Although PTZ binds to the picrotoxin/barbiturate site (Ramajaneyulu & Ticku, 1984), it has also been suggested to act at other sites, such as potassium conductance channels (Gross & Woodbury, 1972) or possibly adrenoceptors (Lazarova *et al.*, 1983).

We have no explanation at present as to why the hypothermic effects of pentobarbitone were slightly increased after kindling, while those of both muscimol and progabide were decreased. Interpretation of the body temperature data is somewhat complicated by the fact that both GABA agonists and antagonists have similar effects; it is difficult to say conclusively that it is a receptor-mediated phenomenon.

It is possible, but unlikely, that the changes in the *in vivo* effects of the drugs studied were due to alterations in their pharmacokinetics. The space of one week between the end of the FG 7142 treatment and the drug tests suggests that an acute interaction of this type was avoided. Whilst long term alterations in pharmacokinetics of the tested drugs cannot be excluded at present, the differences which we found in binding studies suggest that changes in the receptor complex (see below) are a more likely explanation.

We have recently carried out receptor binding studies after chronic FG 7142 treatment, which showed that while there were no consistent changes in binding of [^{3}H]-flunitrazepam or [^{3}H]-βCCE (ethyl-β-carboline-3-carboxylate, an inverse agonist), the GABA stimulation of flunitrazepam binding was consistently reduced (Jeevanjee *et al.*, 1985a). More recently we have examined [^{3}H]-muscimol binding and shown no changes in FG 7142 kindled brains (Martin *et al.*, unpublished observations). However, it is not clear whether this binding reflects the site involved in GABA stimulation of benzodiazepine binding (Olsen,

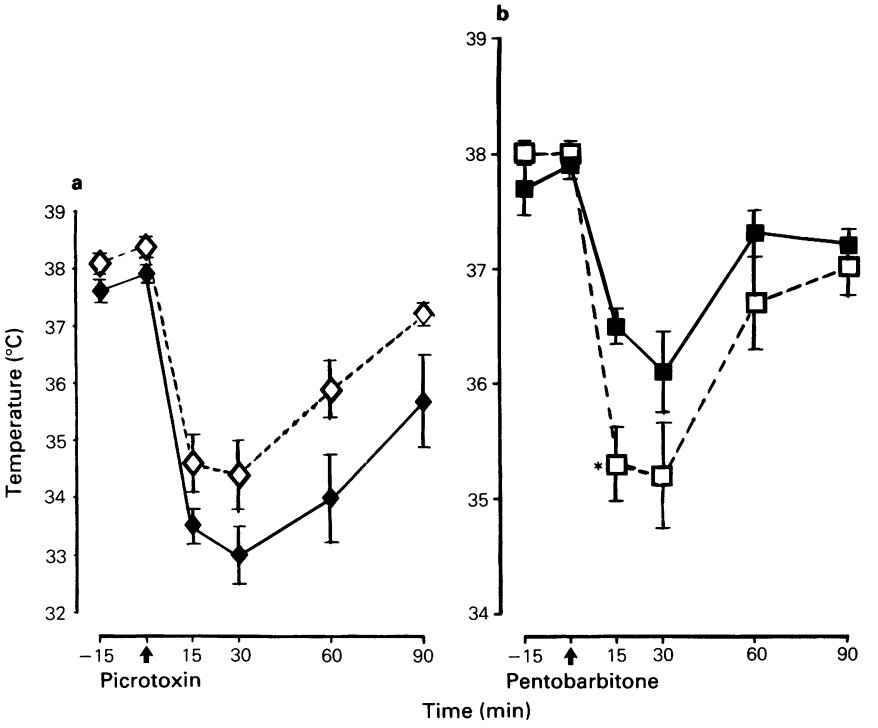

Figure 3 The hypothermic effects of (a) picrotoxin ($2\,mg\,kg^{-1}$) and (b) pentobarbitone ($20\,mg\,kg^{-1}$) in control (injected with Tween vehicle, ◆, ■) FG 7142 kindled (◇, □) mice. Body temperatures were measured at the times shown, before and after intraperitoneal injection of the drugs. Points are mean, with vertical lines indicating s.e.mean, $n = 8$. $^*P < 0.005$.

1981). In contrast it has been shown by other workers that repeated administration of the inverse agonist, βCCE, caused a decrease in the number of [^{3}H]-GABA binding sites in the cortex and hippocampus of rats (Concas *et al.*, 1984).

An overall decrease in GABA transmission *in vivo*, after kindling with FG 7142 would also explain the results from our behavioural studies. We found that mice kindled to FG 7142 showed a decrease in punished responses in the four plate test, in the absence of any acute drug treatment (Jeevanjee *et al.*, 1985b; Little *et al.*, 1986). This effect is thought to represent an increase in anxiety-related behaviour. A similar effect is produced in this test by acute administration of drugs which decrease GABA transmission. The demonstration of such a change, in mice which had received no drug treatment for seven days, suggests a prolonged functional deficit at the GABA-receptor/ionophore complex and this is fully compatible with the decreased effects of GABA agonists shown in the current studies.

We thank the Wellcome Trust for financial support, A/S Ferrosan (Denmark) for supplies of FG 7142 and Synthelabo (France) for the progabide. D.J.N. is a Wellcome Senior Fellow in Clinical Science. S.C.T. is an MRC scholar.

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(Received August 21, 1985.
Revised February 22, 1986.
Accepted March 24, 1986.)