Possible involvement of NMDA receptor-mediated transmission in barbiturate physical dependence

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1 The competitive antagonists at the N-methyl-D-aspartate (NMDA) receptor, CGP39551 and CGP37849, protected against the barbiturate withdrawal syndrome in mice, as measured by ratings of convulsive behaviour on handling.

2 The effective doses of these compounds were lower than those required to prevent seizures due to NMDA in naive animals; these were in turn lower than those needed to prevent the convulsive effects of the α -aminobutyric acid (GABA) antagonist, bicuculline.

3 The NMDA-receptor antagonists did not alter the increase in the incidence of convulsions due to the GABA_A antagonist, bicuculline, that is seen during barbiturate withdrawal, although the latencies to these convulsions during barbital withdrawal were significantly increased after CGP39551.

4 Barbiturate withdrawal did not affect the convulsive actions of NMDA, whether measured by the incidence of convulsions or by intravenous infusion.

5 The $B_{\max}$ for [³H]-dizocilpine ([³H]-MK801) binding was significantly increased by chronic barbital treatment in cerebrocortical but not in hippocampal tissues, while the K_d remained unaltered in either case.

6 At 1 h and 24 h after administration of a single dose of barbitone, the $B_{\max}$ for [³H]-dizocilpine binding was unaltered in cerebrocortical tissue. Acute addition of barbitone *in vitro* did not alter [³H]-dizocilpine binding or the displacement of binding of thienylcyclohexylpyridine.

Keywords: NMDA; barbiturate; withdrawal; binding; dizocilpine; anticonvulsant

Introduction

The barbiturates produce tolerance and a physical dependence that resembles that which develops to ethanol but the physiological changes that are responsible for these effects are not fully understood. Acutely, the barbiturates have actions at several receptor sites or complexes. They are well known to potentiate the effects of γ -aminobutyric acid (GABA), acting at their own receptor sites on the GABA receptor/ionophore complex (Nicoll *et al.*, 1975; Ticku & Olsen, 1978; Macdonald & Barker, 1979). They also block the L-subtype of neuronal calcium channels, and increase inactivation of the N-subtype (Gross & Macdonald, 1988).

Fewer studies have been made on the interactions of barbiturates with excitatory amino acids. The central receptors for these compounds have been divided into subtypes, named after the selective agonists, N-methyl-D-aspartate (NMDA), AMPA (this subtype was previously known as the quisqualate subtype) and kainate. Until recently, antagonists for NMDA receptors were not systemically active, but more lipid soluble compounds are now available, such as CGP39551 or CGP37849 (Fagg *et al.*, 1990; Schmutz *et al.*, 1990). The NMDA receptor complex also bears other receptor sites that interact allosterically, including one that binds glycine, which was found to be an essential co-agonist in the action of NMDA (Johnson & Ascher, 1987).

Early reports described antagonism of responses to glutamate by barbiturates (Richards & Smaje, 1976; Barker & Ransom, 1978; Macdonald & Barker, 1979), and recent papers have examined this effect with respect to the subtypes of excitatory amino acid receptors. The blocking action appears to be selective for the AMPA and kainate receptor subtypes (Teichberg *et al.*, 1984; Sawada & Yamamoto, 1985), although some antagonist actions against aspartate have been reported (Miljkovic & Macdonald, 1986). A recent study (Zeman & Lodge, 1992), in which the effects of low concentrations of kainate were investigated, suggested that

pentobarbitone had a selective blocking action on kainate receptors; higher concentrations of kainate may act at the AMPA receptor. The involvement of excitatory amino acid transmission in barbiturate dependence was suggested by McCaslin & Morgan (1988) who demonstrated that certain antagonists, given intracerebroventricularly, decreased the incidence of abstinence convulsions in rats. The compounds used were 2-amino-7-phosphonoheptanoic acid (APH), that is selective for NMDA sites, glutamyl diethyl ester (GDEE), said to have some selectivity for quisqualate receptors, and *cis*-2,3-piperidine dicarboxylic acid (PDA), a nonselective blocker. The increase in cerebellar guanosine 3':5'-cyclic monophosphate (cyclic GMP) seen during barbiturate withdrawal was decreased by APH, but not by the other compounds. However, it was not clear from this study whether the effect of APH represented a selective action on the withdrawal syndrome, that might suggest an involvement of NMDA in the origin of the seizure state, or a general anticonvulsant action, that might be exerted against convulsions due to any cause.

In the present study we have investigated the effects of the systemically active NMDA antagonists, CGP39551 and CGP37849 on the barbiturate withdrawal syndrome. Because the effects of anticonvulsant agents on withdrawal syndromes are not very informative unless their effects are directly compared with those against other convulsive syndromes (Little, 1991), the actions of these compounds against seizures due to the selective agonist, NMDA, and the GABA antagonist, bicuculline were measured. Their effects on spontaneous locomotor activity were also investigated, in order to see whether or not any effects were due to sedative properties. In order to see whether or not changes occurred at the NMDA receptor after chronic barbiturate treatment, binding of the noncompetitive antagonist, [³H]-dizocilpine was measured.

Preliminary accounts of this work have been presented to the British Pharmacological Society (Rabbani & Little, 1991; Wright *et al.*, 1991).

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Methods

Chronic drug treatment

Barbitone was used for all the chronic barbiturate treatment, because this compound is little metabolized and so does not induce microsomal enzymes. Male mice, TO strain were used, between 20–35 g, with not more than 5 g range in any one experiment and they were housed 10 mice per cage. They were given barbitone in powdered food for 7 days: 3 mg barbitone per g food for 2 days, 4 mg g⁻¹ food for 2 days and 5 mg g⁻¹ food for 3 days. Controls received a matched amount of powdered food only. All mice were weighed regularly during the treatments and no significant differences in weights were found. In all studies the amount of food, and hence barbitone, taken in by the mice was measured daily. The barbitone intake rose during the treatment from 400 mg kg⁻¹ 24 h⁻¹ on the first day to 700 mg kg⁻¹ 24 h⁻¹ on the last day of treatment. For the withdrawal measurements, the mice were placed in clean cages at the end of the 7 day treatment, and given powdered food without barbitone for at least 19 h before testing.

Measurement of the barbiturate withdrawal syndrome

The barbiturate withdrawal syndrome was measured by ratings of convulsive behaviour on handling. This method was first established by Goldstein & Pal (1971) for ethanol withdrawal. Our method was similar to this, with slight modifications (Green *et al.*, 1990). Mice were gently picked up by the tail and turned, first in one direction then the other. Rating numbers were allocated as follows: 0, no signs of tremor or hyperexcitability; 1, occasional signs of tremor; 2, continuous tremor; 3, intermittent clonic convulsions, consisting of repeated contractions of the limbs, particularly the hind legs; 4, continuous clonic convulsions.

The ratings of convulsive behaviour on handling were carried out once an hour, over periods of time after the removal of the barbitone. All the mice were coded so that the observer was unaware of the prior drug treatment. After removal of barbitone from the food, the mice were put into clean cages, to avoid intake of any food in the sawdust. A minimum of 10 animals were used in each treatment group.

The severity of the barbiturate withdrawal syndrome was measured from 20 h to 28 h after barbitone withdrawal. Our previous studies have shown that this period covers the time of maximum changes in behaviour following cessation of the barbitone treatment. Single i.p. injections of CGP39551 and CGP37849 were made at 19 h into the withdrawal period.

Single dose of barbitone

For comparison in the binding studies with the effects of chronic barbitone treatment, the effects of a single intraperitoneal dose of barbitone, or saline, were measured on [³H]-dizocilpine binding. The dose of barbitone used was 250 mg kg⁻¹, which produced moderate ataxia, and the cerebrocortical tissues were prepared at 1 h and at 24 h after the injections.

The effects of bicuculline during barbitone withdrawal

We have previously shown that the convulsive response to bicuculline is increased during barbiturate withdrawal (Rabbani & Little, 1990). The effects of CGP39551 on the actions of bicuculline during barbitone withdrawal were examined at pretreatment times of 1 h and 5 h for CGP39551. The bicuculline was injected i.p., 24 h after barbitone withdrawal and the incidence of seizures noted for the next 45 min. The dose of bicuculline chosen for the barbitone withdrawn mice was 3.5 mg kg⁻¹, which we had previously found to cause clonic convulsions in approximately 90% of mice in these circumstances. For comparison, the effects of the CGP39551

on bicuculline seizures were examined in control mice. Doses of 5 mg kg⁻¹ bicuculline were used in these animals, that gave a response in about 90% of the mice. The effects of CGP37849 against bicuculline convulsions were tested when this compound was given 1 h before the bicuculline administration, with bicuculline given 24 h into withdrawal, and 9 h before bicuculline, when this convulsant was given 28 h into withdrawal (after the handling tests). The mice were killed humanely as soon as the first signs of a convulsion were evident. In all cases the observer was blind to the prior drug-treatment.

Convulsive effects of NMDA

The convulsive effects of NMDA were studied during barbiturate withdrawal by administration of either 250 or 325 mg kg⁻¹ NMDA i.p. 24 h after the withdrawal of the barbitone. The incidence of full seizures, defined as above, and their latencies, were noted for 45 min, by an observer who was not aware of which drug-treatment the animals had received.

The actions of NMDA were also measured by determination of the seizure thresholds by intravenous infusion (Singh *et al.*, 1991). NMDA, dissolved in saline at 50 mg ml⁻¹, was infused into the tail vein at 1.6 ml min⁻¹. The response times for the first muscle twitch, and for the first signs of a full convulsion (which usually took the form of a tonic seizure) were measured by an observer who did not know the prior drug-treatment. Animals were killed humanely as soon as the second endpoint was reached. The seizure thresholds were then calculated in mg kg⁻¹ from the response times and the mouse weights.

Anticonvulsant actions against bicuculline or NMDA in naive mice

The selectivity of the protective effects of the compounds on the barbiturate withdrawal syndrome were examined by their effects on seizures produced by bicuculline, or by NMDA, in naive animals. The doses used of these convulsants, bicuculline 5 mg kg⁻¹ and NMDA 325 mg kg⁻¹, were chosen on the basis of earlier studies, to produce convulsions on 80–90% of a group of mice. The anticonvulsant compounds were given, i.p. 1 h or 5 h before administration of either convulsant, to provide comparison with the withdrawal experiments. The seizure incidence was noted for 40 min following the convulsant injections. A full seizure was recorded when clonic movements of the limbs were observed, accompanied by loss of posture. The mice were killed humanely as soon as the first signs of a convulsion were evident. Statistical comparisons were made only between results from animals tested concurrently. In all cases the observer was blind to the prior drug treatment.

Locomotor activity

The actions of the compounds on spontaneous locomotor activity were measured automatically by breaking of infra-red beams. Pairs of mice, receiving the same drug treatment, were placed in novel cages, and the locomotor activity measured at 5 min intervals for the next 30 min. Six pairs of mice were used for each treatment group. The treatments were randomized throughout the day, between 09 h 00 min and 17 h 00 min, to control for diurnal variations in activity.

[³H]-dizocilpine binding

Following the chronic treatment, the animals were killed by cervical dislocation and the cerebrocortical and hippocampal tissues rapidly dissected out and frozen at -25°C, for a maximum of four weeks. For the studies on cortical tissues, the cortices from two mice were pooled for each experimental

determination, and in the case of hippocampal tissue, the hippocampi from 6 to 8 mice were pooled.

On the day of binding measurement, the frozen tissues were thawed, weighed and suspended in assay buffer (5 mM Tris HCl, pH 7.4 at 25°C) at 55 ml per g of wet tissue, at 0–4°C. They were then hand homogenized, and the homogenate centrifuged at 1,000 g for 10 min. The pellet was discarded and the supernatant was centrifuged at 20,000 g for 20 min. The supernatant was discarded and the pellet was resuspended in the original volume. The suspension was finally centrifuged at 20,000 g for 20 min. The resultant pellet was resuspended in 5 mM Tris HCl to give 0.5 mg ml⁻¹ tissue and kept on ice. The radioligand [³H]-dizocilpine (1.0 mCi ml⁻¹) ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cycloheptane-5,10-imine maleate), was diluted in glass containers with 5 mM Tris HCl, to 1.0 to 15.0 nM. Triplicate determinations were made for each concentration. The non-specific binding was assessed by use of 10 µM cold thienylcyclohexylpiperidine (TCP). Total binding was measured in 1 ml total volume of samples containing: (1) 50 µl assay buffer, 5 mM Tris HCl; (2) 100 µl assay buffer, 5 mM Tris HCl; (3) 100 µl above concentrations of [³H]-dizocilpine; (4) 750 µl tissue aliquot.

Non-specific binding was measured using the following: (1) 50 µl assay buffer, 5 mM Tris HCl; (2) 100 µl TCP at final concentration of 10 µM; (3) 100 µl above concentrations of [³H]-dizocilpine; (4) 750 µl tissue aliquot.

The tubes were gently mixed and placed in a water bath at 25°C for 45 min. Following the incubation of the samples, the tubes were rapidly filtered using 30-place Brandel cell harvester and Whatman GF/B filters. A total of two washes were applied during each filtration, using ice-cold 5 mM Tris HCl. Individual filter papers were removed and placed in vials, then 4 ml of scintillation fluid was added to each vial and counted on the scintillation counter, for 5 min each. In order to measure the amount of radioactivity put into each test tube, 50 µl of the stock and diluted hot [³H]-dizocilpine was added into a scintillation vial and counted as above.

Action of barbitone in vitro on [³H]-dizocilpine binding

In order to check whether or not the barbitone could have had a direct effect on [³H]-dizocilpine binding during the chronic treatment, its actions were tested *in vitro*. The effect on total binding was measured when barbitone was added to the incubation medium at a range of concentrations between 1 nM and 1 mM (at ten fold intervals of concentration). The

effect of barbitone on the displacement of [³H]-dizocilpine by TCP were also studied, by addition of barbitone with each concentration of TCP to barbitone concentrations of 1 and 100 nM, 10 µM and 1 mM. For the saturation experiments, the prepared tissue was incubated as above for 45 min at 25°C, in the presence of either TCP 10 µM or TCP plus barbitone at 1 nM, 1 µM or 100 µM.

Drugs used

CGP39551 (DL-(E)-2-amino-4-methyl-5-phosphonopentanoate carboxy-ethylester, Ciba-Geigy), and CGP37849 (DL-(E)-2-amino-4-methyl-5-phosphonopentanoic acid, Ciba-Geigy) were dissolved in distilled water. Fresh solutions of CGP39551 and CGP37849 were made daily. NMDA was dissolved in saline. Bicuculline was dissolved in glacial acetic acid, brought to pH 3 with NaOH and diluted with saline brought to pH 3 with HCl.

Statistical analysis

The ratings of convulsive behaviour during withdrawal were compared by two-way non-parametric analysis of variance, designed for repeat measurements on the same animals and multiple comparisons with controls (Meddis, 1984). Seizure incidences were compared by Fisher's Exact probability test; comparisons were made only between concurrently tested groups. Comparisons in the measurements of seizure thresholds, latencies and locomotor activity were made by Student's unpaired *t* test. The binding results were analysed using Scatchard analysis, and statistical comparisons made with Student's unpaired *t* test. Either five or six tissue samples, prepared from different animals, with tissues pooled as described above, were used for each set of binding measurements.

Results

Effects of CGP39551 and CGP37849 on the barbiturate withdrawal syndrome

The first experiment showed that CGP39551, at 20 mg kg⁻¹ completely prevented the barbitone withdrawal syndrome

Figure 1 The effects of CGP39551 in decreasing the ratings of convulsive behaviour on handling. Values are medians with representative interquartile ranges. The measurements were made from 19 h to 28 h from cessation of chronic barbitone treatment. (○) Control diet + acute saline; (●) control diet + acute CGP39551, 20 mg kg⁻¹; (□) barbitone diet + acute saline; (■) barbitone diet + acute CGP39551, 20 mg kg⁻¹. The effect of 20 mg kg⁻¹ CGP39551 on the withdrawal hyperexcitability was significant, $P < 0.001$ for comparison between saline and CGP39551 after barbitone treatment, over the whole test period.

Figure 2 The effectiveness of a range of low doses of CGP39551, 1, 2.5 and 5 mg kg⁻¹ against the withdrawal hyperexcitability; the actions of all doses were significant ($P < 0.01$ in each case), when compared with the effects of saline administration, over the whole testing period. (○) Control diet + acute saline; (□) barbitone diet + acute saline; (▲) barbitone diet + acute CGP39551, 2.5 mg kg⁻¹; (■) barbitone diet + acute CGP39551, 5 mg kg⁻¹; (●) barbitone diet + acute CGP39551, 10 mg kg⁻¹. Values are medians with representative interquartile ranges. In all cases the ratings after barbitone withdrawal, with acute saline administration, were significantly higher than in controls that did not receive barbitone ($P < 0.001$).

($P < 0.001$ for comparison between results from barbitone withdrawn animals given saline or CGP39551), over the whole of the testing period (Figure 1). A further study, carried out with lower doses of CGP39551, showed that this compound significantly decreased the withdrawal ratings, at doses of 2.5, 5 or 10 mg kg⁻¹ ($P < 0.01$). These results are illustrated in Figure 2.

CGP37849 also had a protective action on the barbitone withdrawal syndrome, but this was of shorter duration than that of CGP39551 (Figure 3). The effect of the 10 mg kg⁻¹ dose was significant when compared over the first 5 h of testing ($P < 0.05$).

Figure 3 The effects of CGP37849, 10 mg kg⁻¹, on the ratings of convulsive behaviour ($\pm$ interquartile range) during barbitone withdrawal. Values are medians with representative interquartile ranges. (○) Control diet + acute saline; (●) control diet + acute CGP37849, 10 mg kg⁻¹; (□) barbitone diet + acute saline; (■) barbitone diet + acute CGP37849, 10 mg kg⁻¹. The ratings of behaviour after barbitone withdrawal and saline administration were significantly greater than after the control diet and saline administration, as expected. When comparison was made over the first 5 h of testing, this dose of CGP37849 significantly reduced the ratings ($P < 0.005$).

Neither CGP39551 or CGP37849 had any effect on the behavioural ratings in control animals.

Effects of bicuculline during barbitone withdrawal

The responses to bicuculline during barbitone withdrawal are given in Table 1. The administration of 2.5, 5, 10 or 20 mg kg⁻¹ CGP39551, 1 h or 5 h before bicuculline administration, did not alter the incidence of convulsions in response to bicuculline, in either barbitone withdrawn or control mice (Table 1A). The latencies to the convulsions were significantly increased after CGP39551, during barbitone withdrawal only. When the effects of CGP37849 were tested, given 1 h before the administration of bicuculline, there were no significant differences in the responses to bicuculline, either in control animals or those withdrawn from barbitone treatment (Table 1B).

Convulsive effects of NMDA during barbitone withdrawal

The seizure incidence in response to NMDA, shown in Table 2, was unaffected by barbiturate withdrawal. The latencies of the seizures after the 325 mg kg⁻¹ dose were also unaltered. There was a small, but just significant ($P = 0.05$), increase in the latencies after the 250 mg kg⁻¹ dose.

The convulsion thresholds to NMDA measured by the infusion method, are illustrated in Figure 4. There was no significant difference between the thresholds in animals given chronic barbitone treatment and those of controls ($P > 0.1$).

The anticonvulsant actions of CGP39551 and CGP37849 in naive mice

The effects of CGP39551 and CGP37849 against convulsions due to NMDA or to bicuculline were studied in naive mice, for comparison with the effects on the barbiturate withdrawal syndrome. The results are given in Table 3. CGP39551 provided significant protection against NMDA seizures when

Table 1 The actions of CGP39551 and CGP37849 against bicuculline in control and barbitone-withdrawn mice

A CGP39551 was given 1 h or 5 h prior to the bicuculline. In each case the convulsant was given 24 h after the cessation of chronic barbitone treatment

Chronic treatment	Bicuculline dose (mg kg ⁻¹)	Pretreatment time	CGP39551 dose (mg kg ⁻¹)	Seizure incidence	Seizure latencies
Controls	5	1 h	Vehicle	6/8	119 $\pm$ 19
Controls	5	1 h	10	7/8	201 $\pm$ 67
Barbitone	3.5	1 h	Vehicle	7/8	85 $\pm$ 17
Barbitone	3.5	1 h	10	8/8	81 $\pm$ 9
Controls	5	5 h	Vehicle	6/10	168 $\pm$ 46
Controls	5	5 h	20	9/10	178 $\pm$ 42
Barbitone	3.5	5 h	Vehicle	9/9	67 $\pm$ 7
Barbitone	3.5	5 h	5	7/10	110* $\pm$ 27
Barbitone	3.5	5 h	10	8/10	172* $\pm$ 57
Barbitone	3.5	5 h	20	7/9	112* $\pm$ 24

Latencies are mean $\pm$ s.e.mean (s) for the number of mice that convulsed in each group.

* $P < 0.05$ compared with results after vehicle treatment.

B CGP37849 was given 1 h prior to the bicuculline; the convulsant was given 24 h after the cessation of chronic barbitone treatment

Chronic treatment	Bicuculline dose (mg kg ⁻¹)	Pretreatment time	CGP37849 dose (mg kg ⁻¹)	Seizure incidence	Seizure latencies
Controls	5	1 h	Vehicle	6/8	119 $\pm$ 19
Controls	5	1 h	10	8/8	81 $\pm$ 9
Barbitone	3.5	1 h	Vehicle	7/8	85 $\pm$ 17
Barbitone	3.5	1 h	10	6/8	185 $\pm$ 39

Latencies are mean $\pm$ s.e.mean (s) for the number of mice that convulsed in each group.

Figure 4 Convulsion thresholds to N-methyl-D-aspartate (NMDA) during barbitone withdrawal, measured by intravenous infusion of NMDA. Results are expressed as mg kg^{-1} NMDA (mean $\pm$ s.e.mean), the open columns indicate the values for control animals, the hatched columns those obtained during barbiturate withdrawal. The barbiturate withdrawal did not significantly affect either the threshold for the first twitch or the threshold for a full convulsion ($P > 0.1$).

Table 2 Responses to NMDA during barbiturate withdrawal

Chronic treatment	NMDA dose (mg kg^{-1})	Seizure incidence	Seizure latencies (s)
Control	250	5/10	688 $\pm$ 150
Barbitone	250	5/10	1152 $\pm$ 127*
Control	325	6/10	393 $\pm$ 28
Barbitone	325	7/10	513 $\pm$ 99

Latencies are mean $\pm$ s.e.mean (s) for the number of mice that convulsed in each group. Measurements were made 24 h after withdrawal from chronic barbitone treatment. * $P = 0.05$ compared with results after vehicle treatment.

Table 3 Anticonvulsant actions of CGP39551 in naive mice

Convulsant	CGP39551 dose (mg kg^{-1})	Pretreatment time (h)	Seizure incidence	Latencies
NMDA	Saline	1	5/8	754 $\pm$ 122
NMDA	10	1	0/8*	—
NMDA	20	1	0/8*	—
NMDA	Saline	5	7/8	511 $\pm$ 31
NMDA	10	5	5/8	728 $\pm$ 106*
NMDA	Saline	5	8/8	542 $\pm$ 82
NMDA	20	5	2/8*	865 $\pm$ 385
NMDA	Vehicle	1	6/8	886 $\pm$ 170
NMDA	1	1	6/8	839 $\pm$ 110
NMDA	2.5	1	6/8	800 $\pm$ 165
NMDA	5	1	6/8	1009 $\pm$ 196
Bicuculline	Saline	1	4/8	179 $\pm$ 54
Bicuculline	10	1	4/8	118 $\pm$ 17
Bicuculline	20	1	4/8	127 $\pm$ 39
Bicuculline	Saline	5	7/8	121 $\pm$ 19
Bicuculline	10	5	3/8	298 $\pm$ 153
Bicuculline	Saline	5	5/8	171 $\pm$ 79
Bicuculline	20	5	5/8	257 $\pm$ 61

Latencies are mean $\pm$ s.e.mean for the number of mice that convulsed. N-methyl-D-aspartate (NMDA) was given at 325 mg kg^{-1} and bicuculline at 5 mg kg^{-1} . * $P < 0.05$ compared with results after vehicle treatment.

Table 4 Anticonvulsant actions of CGP37849 in naive mice

Convulsant	CGP dose (mg kg^{-1})	Pretreatment time (h)	Seizure incidence	Latencies
NMDA	Saline	1	6/8	675 $\pm$ 122
NMDA	10	1	0/8**	—
NMDA	Saline	5	6/8	572 $\pm$ 158
NMDA	10	5	5/8	1541 $\pm$ 662
NMDA	Saline	5	7/8	511 $\pm$ 31
NMDA	20	5	3/8	939 $\pm$ 54 **
Bicuculline	Saline	1	4/8	195 $\pm$ 34
Bicuculline	10	1	5/8	233 $\pm$ 98
Bicuculline	Saline	1	5/8	171 $\pm$ 79
Bicuculline	20	1	2/8	190 $\pm$ 3
Bicuculline	Saline	5	7/8	121 $\pm$ 19
Bicuculline	10	5	4/8	259 $\pm$ 124
Bicuculline	Saline	5	5/8	171 $\pm$ 79
Bicuculline	20	5	2/8	190 $\pm$ 31

Latencies are mean $\pm$ s.e.mean for the number of mice that convulsed in each group. N-methyl-D-aspartate (NMDA) was given at 325 mg kg^{-1} and bicuculline at 5 mg kg^{-1} . * $P < 0.05$ compared with results after vehicle treatment.

Table 5 The effects of CGP39551 and CGP37849 on spontaneous locomotor activity in naive mice

Drug	Dose (mg kg^{-1})	Pretreatment time (h)	Cumulative activity counts
Saline	—	1	5626 $\pm$ 538
CGP39551	10	1	3837 $\pm$ 809*
CGP37849	10	1	1864 $\pm$ 740**
Saline	—	1	6325 $\pm$ 624
CGP39551	20	1	2466 $\pm$ 550**
CGP37849	20	1	1442 $\pm$ 424**
Saline	—	1	6183 $\pm$ 314
CGP39551	2.5	1	4695 $\pm$ 739
Saline	—	5	6297 $\pm$ 476
CGP39551	10	5	3916 $\pm$ 448**

Cumulative activity was measured for 30 min, starting at the pretreatment times given below. Results are expressed as mean $\pm$ s.e.mean.

* $P < 0.05$, ** $P < 0.01$, compared with concurrently tested controls.

given at 10 mg kg^{-1} , if a 1 h pretreatment time was used but not if given 5 h prior to the convulsant (Table 3). At 20 mg kg^{-1} , this compound decreased the effects of NMDA at both 1 h and 5 h pretreatment times. Neither of these doses of CGP39551, at either pretreatment time, significantly affected the responses to bicuculline. At lower doses, 1, 2.5 or 5 mg kg^{-1} , CGP39551 did not alter the responses to bicuculline (Table 3).

A similar pattern was seen when CGP37849 was used, except that the effects of this compound were of shorter duration, as was found in the withdrawal studies (Table 4). Significant protection against NMDA seizures, but not those due to bicuculline, was seen 1 h after CGP37849. The decrease in seizure incidence, 5 h after the 20 mg kg^{-1} dose of CGP37849, was not significant, although the latencies of the NMDA seizures were significantly increased.

Effects of CGP39551 and CGP37849 on spontaneous locomotor activity

Both CGP39551 and CGP37849, at 10 and 20 mg kg^{-1} , significantly decreased spontaneous locomotor activity in

naive mice (Table 5); CGP37849 appeared to have a greater effect than CGP39551 at each dose, although the differences were not significant ($P > 0.1$). The 2.5 mg kg^{-1} dose of CGP39551, that gave significant protection against the barbiturate withdrawal syndrome, did not affect the spontaneous locomotor activity.

Decreases in spontaneous locomotor activity do not always indicate sedation, but after administration of the above doses of these drugs the animals appeared limp and sleepy and showed reduced responses to external stimuli, such as loud noises.

Figure 5 (a) $B_{\max}$ values measured in tissues prepared 24 h after withdrawal from chronic barbitone treatment. Open columns indicate control values; hatched columns values obtained after chronic barbitone treatment. Values are mean $\pm$ s.e.mean. * $P < 0.05$ for comparison between control and barbitone treatment. (b) K_d values in tissues prepared 24 h after withdrawal from chronic barbitone treatment. Values are mean $\pm$ s.e.mean. Open columns indicate control values; hatched columns values obtained after chronic barbitone treatment. No significant differences were seen.

$[^3\text{H}]$ -dizocilpine binding after chronic barbiturate treatment

The chronic barbiturate administration significantly ($P < 0.05$) increased the $B_{\max}$ values for $[^3\text{H}]$ -dizocilpine binding in cerebrocortical tissue, by 32% (Figure 5a), when the tissues were prepared 24 h after withdrawal of the animals

Figure 6 (a) Specific binding at a range of concentrations of $[^3\text{H}]$ -dizocilpine, measured in cerebrocortical tissue from controls and from mice withdrawn for 24 h from the chronic barbitone treatment: (○) controls; (●) chronic barbitone. Values are mean $\pm$ s.e.mean, from six determinations, using tissues from six animals, in each case. (b) Scatchard plots obtained in the presence and absence of barbitone ($100 \mu\text{M}$) added to the incubation medium *in vitro*: (○) controls; (▲) barbitone. Values are mean $\pm$ s.e.mean, from six determinations in each case. There were no significant differences between the results obtained in the presence and absence of barbitone.

Table 6 Lack of effect of a single dose of barbitone (250 mg kg^{-1}) on K_d and $B_{\max}$ values for $[^3\text{H}]$ -dizocilpine binding in cerebrocortical tissue

Pretreatment time (h)	K_d (nM)		$B_{\max}$ (pmol mg^{-1} protein)	
	Control	Barbitone	Control	Barbitone
1	6.98 ± 1.46	6.68 ± 0.84	6.00 ± 0.68	5.67 ± 0.43
24	4.55 ± 0.33	4.39 ± 0.32	7.31 ± 1.48	6.04 ± 0.85

The effects of a single i.p. dose of barbitone, 250 mg kg^{-1} , on K_d and $B_{\max}$ values for $[^3\text{H}]$ -dizocilpine binding, compared with administration of saline. Results are expressed as mean $\pm$ s.e.mean. There were no significant differences between the values at either 1 h or 24 h after the injections ($n = 6$ per group).

Table 7 Lack of effect of barbitone added *in vitro* on K_d and $B_{\max}$ values for $[^3\text{H}]$ -dizocilpine binding

Barbitone (μM)	K_d (nM)		$B_{\max}$ (pmol mg^{-1} protein)	
	Control	Barbitone	Control	Barbitone
1	6.84 ± 0.90	6.60 ± 0.50	5.73 ± 0.81	5.62 ± 0.69
100	5.91 ± 0.91	5.69 ± 0.79	5.51 ± 0.72	5.40 ± 0.77

The effects of barbitone, 1 and $100 \mu\text{M}$, added *in vitro* on K_d and $B_{\max}$ values for $[^3\text{H}]$ -dizocilpine binding, expressed as mean $\pm$ s.e.mean. There were no significant differences between the groups.

from the treatment. The K_d was not significantly altered (Figure 5b). The mean values calculated for the specific binding in these studies are illustrated in Figure 6a.

In contrast to the results obtained in the cortex, the chronic barbitone treatment left unaltered [3 H]-dizocilpine binding in hippocampal tissues (Figures 5a and b). There were no significant differences in either the B_{max} or the K_d values measured in tissues from controls and barbitone-treated animals ($P < 0.05$).

Administration of a single dose of barbitone

At 1 h and 24 h after administration of 250 mg kg⁻¹ barbitone, the B_{max} and K_d values for binding remained unaltered, compared with those after a vehicle injection. The results are given in Table 6.

Addition of barbitone in vitro

Addition of barbitone to the incubation medium did not change the total binding of [3 H]-dizocilpine, at any of the barbitone concentrations used (results not shown). The displacement of [3 H]-dizocilpine by TCP was not significantly altered by the addition of barbitone, although the mean IC₅₀ values for TCP were lower in each experiment when measured in the presence of barbitone. The K_d and B_{max} values measured in the presence and absence of 1 and 100 μ M barbitone are given in Table 7, and the Scatchard plots obtained during the study of the effects of barbitone, 100 μ M, are illustrated in Figure 6b.

Discussion

The competitive NMDA antagonists, CGP39551 and CGP-37849, prevented all the signs of the barbiturate withdrawal syndrome measured by ratings of convulsive behaviour. This method measures both tremor and convulsions and is the standard method used for estimating withdrawal severity for sedative/hypnotic drugs such as barbiturates and alcohol (Goldstein & Pal, 1971; Green *et al.*, 1990). The method measures the reactions of the animals to a gentle disturbance and the response is characteristic of withdrawal from this type of drug. The effects of the drugs were seen at low doses, down to 2.5 mg kg⁻¹ CGP39551. The effective doses of CGP39551 were lower than those needed to prevent seizures due to NMDA in naive mice. These in turn were lower than the doses of this compound required to prevent seizures caused by the GABA antagonist, bicuculline, in naive mice. A similar pattern was seen in the effects of CGP37849. Decreases in locomotor activity were seen in naive mice after doses of 10 and 20 mg kg⁻¹ of either CGP39551 or CGP37849, but at 2.5 mg kg⁻¹ CGP39551 did not significantly affect locomotor activity. We have previously demonstrated that similarly low doses of CGP39551 and CGP37849 protect against the ethanol withdrawal syndrome (Ripley *et al.*, 1991).

The method of oral administration of barbitone used in this study provides a model of the human oral intake of this type of drug. The intake of barbitone was monitored throughout the chronic treatment and did not differ between cages. Although the exact doses received by individual mice were not known precisely, the animals were randomly allocated to acute treatment groups at the beginning of the withdrawal measurements and it was clear from the results that all the mice received sufficient drug for a sufficient length of time to produce a withdrawal syndrome.

The effectiveness of the NMDA antagonists in preventing the barbiturate withdrawal signs raised the possibility that changes at the NMDA receptor complex may be involved in the genesis of the withdrawal hyperexcitability. Evidence that neurochemical changes do occur at this receptor complex was provided by the demonstration that the density of NMDA

receptors, measured by binding to the noncompetitive antagonist dizocilpine, was increased after the chronic barbitone treatment.

The selectivity of the action of the NMDA antagonists during barbiturate withdrawal, and the low doses required to produce the protective effect, strongly suggested that the underlying causes of the withdrawal hyperexcitability involve increased activity at NMDA receptors. However, the convulsive effects of NMDA were unaltered during barbiturate withdrawal, despite the increase in binding site number.

In the present study, the distinction between the doses of CGP39551 and CGP37849 that were effective against barbiturate withdrawal hyperexcitability and those with anticonvulsant action in other models was clear, but protection against other types of seizure has been reported at the former doses. Antagonism of NMDA receptor activity is well-established to have anticonvulsant actions in many models, although much of the work so far has been on the non-competitive antagonist, dizocilpine. Schmutz *et al.* (1990) demonstrated protective actions of CGP39551 and CGP-37849 against maximal electroshock seizures in rats, with ED₅₀ values of 2.8 mg kg⁻¹ and 2.0 mg kg⁻¹, respectively, after intraperitoneal administration. The times at which the effects were maximal were found to be 4 h for CGP39551 and 2 h for CGP37849. In our animals, the protective effects of CGP39551 against NMDA seizures in naive mice were clearly greater at 1 h than at 5 h after treatment. Fagg *et al.* (1990) showed the competitive antagonists used in the present study to be effective against electroshock seizures, with oral ED₅₀ values of 4 mg kg⁻¹ for CGP39551 and 21 mg kg⁻¹ for CGP37849. Comparison between these values and those used in the present study is difficult because of the different route of administration, and the fact that differences between the two compounds in bioavailability have been suggested (Loscher & Honack, 1991). The latter authors studied the effects of CGP39551 and CGP37849 on kindled seizures in rats, when these compounds were given by the intraperitoneal route at 1, 2.5 and 5 mg kg⁻¹. At these doses, both compounds were ineffective against kindled seizures in this model. In the actions of the competitive NMDA antagonists, therefore, the barbiturate withdrawal syndrome and that of ethanol, resemble maximal electroshock seizures, rather than kindled seizures. Kindled seizures are regarded as a model for complex partial epilepsy, while the effects of drugs against maximal electroshock resemble those against generalized tonic-clonic seizure states in humans. The underlying causes of maximal electroshock seizures are unclear, but are likely to include increased NMDA receptor activity.

Loscher & Honack (1991) described excitatory actions of CGP39551 and CGP37849 in the kindled rats, including increased locomotor activity, head-weaving and circling. These were attributed to an increased function of NMDA receptors reported to occur after kindling (Mody & Heinemann, 1987), as they are not seen in nonkindled animals. None of these behaviours was seen in mice during barbiturate withdrawal in the present study, even though upregulation of NMDA receptor sites occurred after the barbiturate chronic treatment. The evidence for increased NMDA receptor activity during the ethanol withdrawal syndrome is greater than for barbiturate withdrawal, as many more studies have been carried out (see Grant *et al.*, 1990; Whittington *et al.*, 1992; and the review, Little, 1991), but no such abnormal behaviours were seen with CGP39551 or CGP37849 during our studies on this syndrome.

The chronic barbitone treatment caused an increase in the density of binding to [3 H]-dizocilpine in cerebocortical tissue but not in the hippocampus. This ligand acts at the non-competitive site on the NMDA complex and was used as a marker for NMDA receptor. As described in the introduction, the acute action of barbiturates is to block responses at the AMPA and kainate subtypes of receptor, with less effect at NMDA receptors. This differential effect has been described in both hippocampal (Sawada & Yamamoto, 1985;

Miljkovic & Macdonald, 1986) and cortical tissue (Harrison & Simmonds, 1985), as well as in striatal preparations (Teichberg *et al.*, 1984). Barbitone added *in vitro*, over a wide range of concentrations, did not alter [³H]-dizocilpine binding. The upregulation of the number of NMDA receptors in the cerebral cortex after chronic treatment may therefore represent the involvement of these receptors in neuronal plasticity, rather than a direct adaptive response to initial blockade. Upregulation of [³H]-dizocilpine binding has also been seen after chronic phenobarbitone administration (Short & Tabakoff, 1993) and after chronic ethanol treatment (Grant *et al.*, 1990).

No upregulation of [³H]-dizocilpine binding was seen in hippocampal tissue after chronic barbitone treatment. It has not been established whether the hyperexcitability seen during barbiturate withdrawal originates in one area of the CNS or if many areas become hyperexcitable simultaneously. Hinman & Okamoto (1984) recorded EEG patterns *in vivo* during 'moderate intensity' barbiturate withdrawal. Rhythmical burst of activity were seen in the hippocampus, amygdala and visual cortex and spike and wave activity in the hippocampus, accompanied by burst activity in the motor cortex

when spontaneous generalized seizures occurred. These data suggest that both the hippocampus and cortical areas are involved in the withdrawal syndrome, but the patterns of adaptation may vary in different brain areas.

In conclusion, the evidence from the behavioural studies, demonstrating a potent protective effect of NMDA-receptor antagonists against withdrawal signs, and the receptor binding studies, showing upregulation of dizocilpine binding sites, suggests that increased activity at the NMDA receptors may contribute to the physical signs of withdrawal from chronic barbitone treatment. However, the lack of effect of the NMDA antagonists on the increased sensitivity to bicuculline-induced convulsions during the withdrawal period, and the lack of increased sensitivity to NMDA seen at this time show that other factors may be involved.

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