Chronic dihydropyridine treatment can reverse the behavioural consequences of and prevent adaptations to, chronic ethanol treatment

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- 1 Chronic treatment with the dihydropyridine calcium channel antagonist, nitrendipine, given concurrently with ethanol, prevented the ethanol withdrawal syndrome in mice, even though the chronic nitrendipine treatment was stopped 24 h or 48 h before the withdrawal testing.
- 2 This effect was seen in two strains of mice with different methods of ethanol administration. Nitrendipine was effective when given for two weeks but not after only two days' treatment.
- 3 Two other dihydropyridine calcium antagonists, nimodipine and PN 200-110, given chronically with ethanol, also prevented the withdrawal syndrome. The tests were again made 24 h after the last administration of dihydropyridine.
- 4 The chronic nitrendipine treatment also prevented the rise in the number of central dihydropyridine binding sites that occurs on chronic ethanol administration.
- 5 Chronic administration of nitrendipine alone did not cause any withdrawal behaviour.
- 6 Chronic nitrendipine treatment did not affect the seizure threshold to bicuculline in mice that were not given ethanol.
- 7 Whole brain concentration measurements showed that the effects were not due to residual nitrendipine in the CNS at the time of withdrawal testing or to differences in central ethanol concentrations during the treatment.
- 8 It is suggested that the results provide evidence for a functional role for dihydropyridine-sensitive calcium channels in ethanol dependence.

Keywords: Ethanol withdrawal; dihydropyridines; calcium; chronic ethanol treatment

Introduction

The neurochemical basis of dependence on ethanol is uncertain. Ethanol affects a wide range of biochemical functions and many different neurotransmitters. Its effects may be due to actions on membrane lipids (Goldstein, 1986), which could result in the observed alterations in neuronal transmission. Tolerance occurs to many of its effects on long term treatment and adaptations have been demonstrated in many different systems. However, so far no single action or adaptation has been shown to be responsible for ethanol-dependence.

Withdrawal from chronic ethanol intake results in a hyperexcitability syndrome, seen in animals as tremor, hyperreflexia and convulsions, described by Goldstein & Pal (1971). In man similar symptoms occur, plus confusional states and hallucinations. Drugs that have similar actions to ethanol, such as the benzodiazepines or barbiturates, will substitute for ethanol in withdrawal, and are used therapeutically to control the syndrome. However, these compounds possess dependence liability of their own and do not appear to alter the underlying changes responsible for ethanol-dependence.

We have found that chronic administration of ethanol *in vivo* increases the number of dihydropyridine binding sites in the central nervous system (Dolin *et al.*, 1987). When a single strain of rodents was used, inbred for either ethanol withdrawal sensitivity or resistance, the increase in dihydropyridine binding sites after ethanol treatment correlated well with withdrawal severity (Brennan *et al.*, 1990). These binding sites show a high affinity for dihydropyridine calcium channel antagonists, such as nifedipine or nitrendipine, and the calcium channel activators, such as Bay K 8644 (Littleton & Little, 1987), but their physiological role is uncertain (Miller, 1985). They may correspond to the 'L' subtype of neuronal

calcium channel, described in cultured cells of neuronal origin (Nowicky *et al.*, 1985), although the selectivity for this subtype may not be as clear in mammalian neurones (Akaike *et al.*, 1989; Takahashi *et al.*, 1989; Takahashi & Akaike, 1990). Dihydropyridine calcium channel antagonists have few overt behavioural actions in normal circumstances, although high affinity binding sites have been identified in the CNS (Murphy *et al.*, 1982; Schoemaker *et al.*, 1983). They were originally thought to have little effect on neurones, but actions on central neurones are being increasingly reported (Middlemiss & Spedding, 1986; Raeburn & Gonzales, 1988; Grover & Teyler, 1990). The increase in binding site number found after chronic ethanol administration was accompanied by increases in the actions of the dihydropyridines on transmitter release and on inositol phospholipid breakdown (Dolin *et al.*, 1987), suggesting a functional change caused by the ethanol.

Calcium channel antagonists, given acutely at the start of ethanol withdrawal, decreased the ethanol withdrawal syndrome (Little *et al.*, 1986). This action was stereospecific (Littleton *et al.*, 1990), being seen with the (+)-isomer of PN 200-110, but not with the (–)-isomer that does not block calcium channels (Hof *et al.*, 1986). The effect appeared to be selective for the ethanol withdrawal syndrome as dihydropyridines, such as nitrendipine and nimodipine, were found to provide little protection against other convulsive states (Dolin *et al.*, 1988a). We have found no anticonvulsant action against the GABA_A antagonist, bicuculline (Dolin *et al.*, 1990). When administered with ethanol, dihydropyridine calcium channel antagonists potentiated the actions of ethanol (Dolin & Little, 1986).

From these results we put forward the hypothesis that some of the changes that occur on chronic ethanol intake could be due to the increase in number and function of dihydropyridine binding sites (Littleton *et al.*, 1988; Dolin & Little, 1989). If

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this hypothesis were correct, then prevention of the increase in number of dihydropyridine-sensitive sites should prevent the adaptations to chronic ethanol intake. In 1985, Panza *et al.* reported that chronic administration of a dihydropyridine calcium channel antagonist resulted in a decrease in the number of central dihydropyridine binding sites. The present study was designed to determine whether chronic administration of a dihydropyridine would alter the adaptations to long term ethanol administration, as manifest by the ethanol withdrawal syndrome. Comparison was made with the effects of such chronic dihydropyridine treatment on seizure thresholds to bicuculline, in order to determine the selectivity of protection against the ethanol withdrawal seizure activity.

Some of these results have been presented in abstract form to the British Pharmacological Society (Dolin *et al.*, 1988b).

Methods

Chronic drug administration

Two methods of chronic ethanol administration were used, and two strains of mice. This avoided any influence of the method of administration or the rodent strain on the results. Male mice, C57 strain (22–27 g at the start of the treatment) were given ethanol, 24% v/v in tap water, as sole fluid for twelve weeks. Nitrendipine was given either by injection (see below) or dissolved in the drinking fluid at 1.77 mM. This concentration was the highest that would remain in solution in the drinking bottles. Nitrendipine was added to the drinking fluid either for the whole of the twelve weeks ethanol treatment period or for the last two weeks of this period. The amount of fluid drunk by each treatment group in every experiment was measured three times a week. No significant differences were found between the amount of ethanol drunk by mice receiving concurrent nitrendipine and those given ethanol alone. The ethanol intake values when nitrendipine was dissolved in the ethanol throughout the treatment period, for example, were: ethanol alone, $13.5 \pm 0.4 \text{ g kg}^{-1}$ daily and ethanol plus nitrendipine, $13.3 \pm 0.3 \text{ g kg}^{-1}$ daily (mean $\pm$ s.e.mean, 11 mice in each group). In both studies, nitrendipine was removed from the drinking fluid 24 h before withdrawal of ethanol and behavioural rating (see below). Controls drank tap water. The dose of nitrendipine received by this method of administration was $51 \pm 5 \text{ mg kg}^{-1}$ daily (mean $\pm$ s.e.mean for both experiments, 23 mice).

In the second method, male mice of the TO strain (25–30 g) were kept in inhalational chambers for two weeks in an atmosphere of $12\text{--}18 \text{ g l}^{-1}$ ethanol. The ethanol concentrations in the chambers were measured twice daily. Groups of mice that were to undergo comparison in behavioural experiments were always kept in the same chamber. Controls were kept in the same environment, outside the chambers.

Nitrendipine was suspended in Tween 80, 0.5%, and sonicated well before use. As the drug is photosensitive, it was protected from light at all times by use of a red safe-light and dark bottles. In the injection schedules, mice were given injections, i.p., twice daily (10 h 00 min and 22 h 00 min) of either nitrendipine, 50 mg kg^{-1} or 10 mg kg^{-1} , or the vehicle, Tween 80, 0.5%. The choice of doses of nitrendipine was based on our previous work on the interactions between ethanol and nitrendipine (Dolin & Little, 1989) and the effects of nitrendipine in the ethanol withdrawal syndrome (Littleton *et al.*, 1990). The last injections were given 24 h before the testing of the withdrawal syndrome, unless otherwise stated.

All groups of animals remained healthy throughout the treatments. They were weighed at regular intervals during the treatments and there were no significant differences between the weight gains of the different groups. The numbers of mice in each treatment group were between 7 and 12.

The details of the duration of the nitrendipine treatments are given in each results section. In all cases the nitrendipine

treatment was stopped at least 24 h before the withdrawal testing. For the drinking schedules, the nitrendipine was removed from the drinking fluid 24 h before the withdrawal of ethanol; for the injection schedules the last injections were given at this 24 h interval. This was because the intention of the experiments was to study the effects of the chronic treatments, not the acute action of nitrendipine on the withdrawal syndrome.

The effects of two weeks' administration of nitrendipine, 50 mg kg^{-1} twice daily, by injections, in the absence of ethanol was examined in the TO strain of mice. It was not possible in the C57 mice, where the ethanol was given in the drinking fluid, to make a direct comparison with the effects of nitrendipine alone dissolved in the drinking fluid, because nitrendipine was not sufficiently soluble to dissolve in water without ethanol.

In separate experiments the effects on the ethanol withdrawal syndrome of chronic treatment with two other dihydropyridine compounds were examined. Nimodipine was given twice daily at 50 mg kg^{-1} and ($\pm$)-PN 200-110 twice daily at 4 mg kg^{-1} . In each case the last injection was given 24 h before withdrawal of ethanol.

The ethanol withdrawal syndrome

The ethanol withdrawal syndrome was measured by ratings of convulsive behaviour (Goldstein & Pal, 1971; Littleton *et al.*, 1990). This method measures both tremor and clonic convulsions. The mice were removed from the ethanol between 09 h 00 min and 09 h 30 min. Behavioural ratings were made once an hour on all mice for 12 h.

The observer who carried out the ratings was unaware of the prior drug treatment. The ratings of the withdrawal syndrome were compared by nonparametric analysis of variance, using a format designed for repeat measurements on the same samples and multiple comparisons with controls (Meddis, 1984). Statistical comparisons were made on the ratings at between 3 h and 12 h from the start of withdrawal. During the first 2 h the withdrawal syndrome was beginning, but was not seen clearly until about 3 h.

Dihydropyridine binding

Central dihydropyridine receptor binding was measured by the method of Glossman & Ferry (1985), using [^3H]-nimodipine (specific activity 130 Ci mmol^{-1}). The tissues were homogenized in Tris buffer, 50 mM and centrifuged ($45,000 \text{ g}$, 15 min) and washed with the buffer three times. Tissue aliquots were incubated (25°C , 45 min) with [^3H]-nimodipine, 0.125 nM to 4 nM, then filtered (Whatman GF/B glass filters) and counted. All measurements were made in triplicate. Non-specific binding was estimated with $1 \mu\text{M}$ nitrendipine. The protein content of the tissues was measured with Folin's reagent. The results were subjected to Scatchard analysis to give the receptor density, B_{max} , in fmol mg^{-1} protein, and the dissociation constant, K_d in nM.

The measurements of [^3H]-nimodipine binding were made in whole brains of C57 mice and in cerebral cortex of TO mice. The C57 tissues were taken at 5 h from ethanol withdrawal, when the effects of the drug treatment were evident in the parallel behavioural tests. Nitrendipine was given by i.p. injection at 50 mg kg^{-1} , twice daily, for the last two weeks of the ethanol drinking period. Animals receiving ethanol alone and controls were given vehicle injections. The TO mice received two weeks inhalation of ethanol, as described above, with injections of nitrendipine, 50 mg kg^{-1} , or vehicle, twice daily. In both studies the last injections were given 24 h before removal of tissues.

Measurement of seizure threshold to bicuculline

For comparison with the ethanol withdrawal experiments, seizure thresholds to bicuculline were determined after two

weeks treatment with nitrendipine, twice daily as above, in TO mice that did not receive ethanol. The amount of bicuculline required to cause the first signs of clonic convulsion was measured, by the tail vein infusion method of Nutt *et al.* (1980). The mice were humanely killed immediately the first sign of a seizure was seen. These measurements were made between 30 h and 32 h after the last of the chronic treatment injections, to correspond to the time at which a clear drug effect was seen in the ethanol withdrawal studies. The *n* values were 7 for the controls and 9 for the nitrendipine treatment.

Measurement of central nitrendipine concentrations

Measurements were made of the concentrations of nitrendipine in brains removed from animals receiving the chronic treatment. The brains were removed, following cervical dislocation, from C57 mice, that had been drinking the ethanol and nitrendipine solution for 12 weeks as described above. Tissues were taken at the end of the chronic treatment (at 10 h 00 min before removal of the nitrendipine) and 32 h after removal of nitrendipine from the drinking fluid, i.e. 8 h after withdrawal of ethanol, to correspond with the peak drug effect in the withdrawal testing. Comparison was made with the brain concentrations 2 h and 8 h after acute injections of nitrendipine, in C57 mice that had been drinking 24% ethanol alone for twelve weeks.

Whole brain nitrendipine concentrations were measured by gas/liquid chromatography. Electron capture detection was used to analyse samples separated with a 25 meter capillary column coated with SE30. The temperature programming for the analysis was as follows: injector 280°C, detector 320°C, oven started at 180°C for 2 min then ramped up to 290°C at 16°C min⁻¹ and left at this temperature for 10 min. Helium carrier gas at 6 p.s.i. was used to ensure the flow of sample along the column at the optimum speed for separation. The sample was flushed from the end of the capillary column into the detector with nitrogen make-up gas at 20 p.s.i.

The brains were homogenized in 10 M NaOH, 1 ml per 50 mg wet weight, and the proteins precipitated with sodium tungstate and sulphuric acid. The samples were centrifuged at 4000 r.p.m. for 10 min, then the nitrendipine was extracted by shaking with the same volume of toluene. The organic layer was removed and dried with anhydrous MgSO₄; then the samples were evaporated to dryness and reconstituted in 100 µl toluene, with 1 µM nimodipine as internal standard.

Brain concentrations of ethanol

The brain concentrations of ethanol were measured at times corresponding to the peak drug effect in the behavioural tests to ensure that the chronic treatments did not alter the pharmacokinetics of ethanol. The samples were taken at 2 h from ethanol withdrawal for the C57 strain (drinking ethanol or ethanol plus nitrendipine) and 5 h from withdrawal for the TO strain (ethanol inhalation, with and without nitrendipine injections), after the chronic treatment schedules described above.

Ethanol concentrations were measured by gas-liquid chromatography, with flame ionization detection, Pye type 104 dual column. Whole brains were homogenized in 150 mM NaCl, diluted 1:3, and the internal standard, n-propanol (100 µg ml⁻¹) added. Proteins were precipitated with sodium tungstate and sulphuric acid. The areas under the curves were compared; recovery of ethanol was 100%.

Brain concentrations of ethanol, the results from the binding studies and the ethanol consumption of the groups of C57 mice, were compared by Student's *t* test.

Drugs used

Nitrendipine and nimodipine were kindly donated by Bayer A/G. (±)-PN 200-110 (isopropyl-4- (2,1,3-benzoxadiazol-4-yl-

1,4-dihydro-2,6-dimethyl-5-methoxy-carbonyl-pyridine-3-carboxylate) was given by Sandoz.

Results

Effects of nitrendipine on the withdrawal syndrome in C57 mice drinking ethanol

Nitrendipine given in the drinking fluid for 12 weeks Removal of ethanol resulted in the appearance of a clear ethanol withdrawal syndrome, beginning approximately 2–3 h after withdrawal (Figures 1a and b). When nitrendipine was given for the 12 week ethanol drinking period, this withdrawal syndrome was not seen (Figure 1a). The ratings of convulsive behaviour were significantly lower than those for animals given ethanol alone ($P < 0.005$) and were not significantly different from control values, i.e. from ratings of animals which did not receive any ethanol ($P > 0.1$).

Nitrendipine dissolved in the drinking fluid for 2 weeks of the 12 week drinking period When nitrendipine was given for the last 2 weeks only of the 12 weeks of ethanol drinking there was a significant decrease in the withdrawal ratings ($P < 0.05$), compared with those for animals given ethanol alone, but the syndrome was not completely abolished (Figure 1b). The ratings for mice given ethanol plus nitrendipine were significantly higher ($P < 0.05$) than those of control animals that were not given ethanol.

Nitrendipine given by injection for 2 weeks to C57 mice drinking ethanol The ethanol withdrawal syndrome was also prevented when nitrendipine was given to C57 mice by injection (50 mg kg⁻¹, twice daily) for the last 2 weeks of the 12 week drinking period, up until the last 24 h of ethanol treatment (data not shown). The ratings of withdrawal after the nitrendipine treatment were significantly lower ($P < 0.001$) after nitrendipine plus ethanol than after ethanol alone, and were not significantly different from control values ($P > 0.1$).

Effects of nitrendipine on TO mice given ethanol by inhalation

Nitrendipine given twice daily for 2 weeks at 50 mg kg⁻¹ Removal of mice from the ethanol inhalational chamber resulted in the appearance of a withdrawal syndrome similar to that seen in the C57 mice, although the ratings were slightly lower. Injections of nitrendipine for the whole 2 weeks of the ethanol administration abolished the syndrome (Figure 2a). The difference between the ratings for animals given ethanol alone and those given ethanol plus nitrendipine was significant ($P < 0.001$) and the latter ratings were not different from control values ($P > 0.1$). In this experiment the last injection of nitrendipine was made 24 h before the withdrawal testing. When the last injection was made 48 h before the withdrawal the effect of nitrendipine was still seen (Figure 2b) ($P < 0.001$ for comparison between ethanol alone and ethanol plus nitrendipine).

Effect of duration of nitrendipine administration In a separate experiment, nitrendipine was given, at 50 mg kg⁻¹, to TO mice for 2 days only, during the 48 h preceding the 24 h interval before the withdrawal of ethanol (i.e. four injections, the last being given 24 h before withdrawal). In this study there was no effect of nitrendipine ($P > 0.1$) on the withdrawal syndrome (Figure 3a).

Dose-dependency of nitrendipine action The administration of nitrendipine at 10 mg kg⁻¹ twice daily during the 2 week ethanol administration period caused a significant decrease in

Figure 1 (a) The effects of either ethanol alone or ethanol plus nitrendipine (1.77 mM) given to C57 mice in the drinking fluid for 12 weeks. The addition of nitrendipine abolished the withdrawal syndrome, even though little drug remained in the CNS at the time of testing. Results in this and subsequent figures are median values ($\pm$ interquartile ranges) for 7–12 mice. The ratings of convulsive behaviour were significantly lower in animals given nitrendipine injections than those for animals given ethanol alone ($P = 0.005$) and were not significantly different from control values, i.e. from ratings of animals which did not receive any ethanol ($P > 0.1$). Comparisons made between 3 h and 12 h of the testing period: ($\blacktriangle$) ethanol plus Tween vehicle injections; ($\blacksquare$) ethanol plus nitrendipine injections; ($\square$) controls. (b) Nitrendipine dissolved in the drinking fluid for the last 2 weeks of the 12 week drinking period decreased with withdrawal syndrome in C57 mice. There was a significant decrease in the withdrawal ratings ($P < 0.05$), for animals given nitrendipine compared with those for animals given ethanol alone. The ratings for mice given ethanol plus nitrendipine were significantly higher ($P < 0.05$) than those of control animals not given ethanol: ($\blacktriangle$) ethanol alone; ($\blacksquare$) ethanol plus nitrendipine; ($\square$) controls.

the withdrawal ratings, compared with mice given ethanol alone, $P < 0.001$ (Figure 3b). However, the withdrawal syndrome was not completely abolished, as had been seen with the 50 mg kg^{-1} dose of nitrendipine, as the ratings were significantly higher than those of control animals, given no ethanol, $P < 0.001$.

The effects of chronic treatment with the dihydropyridines nimodipine and PN 200-110 Chronic nimodipine treatment also prevented the withdrawal syndrome to ethanol (Figure 4a). The ratings after ethanol plus nimodipine were significantly lower than those after ethanol alone ($P = 0.005$), and were not significantly different from control values. A similar, but smaller, effect was seen with PN 200-110 (Figure 4b). In this case the ratings after ethanol plus PN 200-110 were signifi-

Figure 2 Nitrendipine given twice daily at 50 mg kg^{-1} for the 2 week period of ethanol inhalation abolished the withdrawal syndrome in the TO strain of mice. The difference between the ratings for animals given ethanol alone and those given ethanol plus nitrendipine was significant ($P < 0.001$) and the latter ratings were not different from control values ($P > 0.1$). The last injections were given 24 h before the tests were commenced: ($\blacktriangle$) ethanol plus Tween vehicle injections; ($\blacksquare$) ethanol plus nitrendipine injections; ($\square$) controls. (b) As in (a) except that the last injections were made 48 h before the withdrawal. The effect of nitrendipine was still seen, $P < 0.001$ for comparison between ethanol alone and ethanol plus nitrendipine: ($\blacktriangle$) ethanol plus Tween vehicle injections; ($\blacksquare$) ethanol plus nitrendipine injections; ($\square$) controls.

cantly lower than those after ethanol alone ($P < 0.01$), but were higher than the control values ($P < 0.05$).

Nitrendipine in the absence of ethanol treatment The administration of nitrendipine at 50 mg kg^{-1} , twice daily, did not result in the appearance of any withdrawal signs in mice that did not receive any ethanol, in either the TO strain or the C57 strain (not illustrated). The ratings of convulsive behaviour were not significantly different from those of mice that were given vehicle injections, $P > 0.1$.

Central dihydropyridine binding

The values for dihydropyridine binding are given in Table 1. In tissues from both C57 mice and the TO strain there was a significant increase in the B_{max} values when comparison was made between ethanol treatment and controls ($P < 0.05$), but this difference was not significant ($P < 0.1$) when the B_{max} values for ethanol plus nitrendipine treatment were compared with controls. The same pattern was therefore seen in both sets of binding data even though different strains of mice were

Figure 3 (a) When nitrendipine was given to TO mice for the last 2 days only of the 2 week ethanol administration period, at 50 mg kg^{-1} , there was no effect on the withdrawal syndrome, $P > 0.1$ for comparison between ethanol alone and ethanol plus nitrendipine: (▲) ethanol plus Tween vehicle injections; (■) ethanol plus nitrendipine injections; (□) controls. (b) The administration of nitrendipine at 10 mg kg^{-1} twice daily during the 2 week ethanol administration period caused a significant decrease in the withdrawal ratings, compared with mice given ethanol alone, $P < 0.001$. Ratings for ethanol plus nitrendipine treatment were higher than those of control animals, given no ethanol, $P < 0.001$: (▲) ethanol plus Tween vehicle injections; (■) ethanol plus nitrendipine injections; (□) controls.

used, different areas (whole brain and cerebral cortex) and the tissues were removed at different time periods (5 h from ethanol withdrawal and immediately on withdrawal). There were no significant differences in K_d values.

Table 1 B_{max} and K_d values for [^3H]-nimodipine binding in C57 and TO mice, taken 5 h and 0 h, respectively, from withdrawal of ethanol

Chronic treatment	B_{max} (fmol mg^{-1})	K_d (nM)
(i) C57 mice, 5 h from ethanol withdrawal		
Controls, vehicle injections (11)	232 ± 10	0.67 ± 0.07
Ethanol alone, 12 weeks (9)	$277^* \pm 16$	0.74 ± 0.07
Ethanol, 12 weeks, plus 2 weeks injections nitrendipine (7)	249 ± 17	0.74 ± 0.04
(ii) TO mice, 0 h from withdrawal		
Controls, vehicle injections (5)	230 ± 20	0.99 ± 0.27
Ethanol alone, vehicle injections (5)	$300^* \pm 18$	0.93 ± 0.09
Ethanol plus nitrendipine injections for the whole 2 weeks (5)	253 ± 18	0.79 ± 0.06
Nitrendipine injections alone (5)	210 ± 21	0.67 ± 0.04

Values are mean $\pm$ s.e.mean; n is given in parentheses.

* $P < 0.05$, Student's t test, compared with control values.

Figure 4 (a) Nimodipine, twice daily at 50 mg kg^{-1} , for the 2 week period of ethanol inhalation, prevented the withdrawal syndrome in the TO strain of mice. The difference between the ratings for animals given ethanol alone and those given ethanol plus nimodipine was significant ($P < 0.005$) and the latter ratings were not different from control values ($P > 0.1$). The last injections were given 24 h before the tests were commenced: (▲) ethanol plus Tween vehicle injections; (●) ethanol plus nimodipine injections; (□) controls. (b) PN 200-110, at 4 mg kg^{-1} , twice daily for the 2 week period of ethanol inhalation, abolished the withdrawal syndrome in the TO mice. The ratings for animals given ethanol plus PN 200-110 were significantly different from those following ethanol alone ($P < 0.01$) and from control values ($P < 0.05$). The last injections were given 24 h before the tests were commenced: (▲) ethanol plus Tween vehicle injections; (●) ethanol plus nitrendipine injections; (□) controls.

Lack of effect of nitrendipine treatment on seizure thresholds to bicuculline

The chronic nitrendipine treatment had no effect on the seizure thresholds to bicuculline in naive mice, i.e. animals that were not given any ethanol ($P > 0.1$). The thresholds were $0.32 \pm 0.07 \text{ mg kg}^{-1}$ bicuculline for the controls and $0.35 \pm 0.09 \text{ mg kg}^{-1}$ bicuculline for the group given nitrendipine treatment. The time of measurement of these thresholds after the end of the chronic treatment schedule corresponded to the peak drug effect in the ethanol withdrawal studies.

Central nitrendipine concentrations

The whole brain concentrations of nitrendipine are given in Table 2. The concentrations at the time of clear drug effects in the withdrawal testing (8 h withdrawal from ethanol, 32 h withdrawal from nitrendipine) were considerably lower than those achieved following acute injections of nitrendipine.

Table 2 A Brain concentrations of nitrendipine in C57 mice after 12 weeks drinking ethanol plus 1.77 mM nitrendipine

Time	Nitrendipine concentration (ng g ⁻¹ wet wt.)
(a) During treatment	248 ± 32 (6)
(b) Prior to withdrawal	64 ± 11 (6)
(c) During withdrawal	55 ± 11 (6)

Tissues were taken: (a) At the end of the chronic treatment, before removal of nitrendipine from the drinking fluid; (b) Just before withdrawal from the ethanol, 24 h after removal of nitrendipine from the drinking fluid; (c) At a time corresponding to a clear drug effect on the withdrawal syndrome, 32 h after removal of nitrendipine from the drinking fluid and 8 h from ethanol withdrawal.

B Brain nitrendipine concentrations in C57 mice after acute injections of nitrendipine, given immediately on withdrawal from twelve weeks drinking ethanol.

Nitrendipine dose	Time from injection	
	2 h	8 h
10 mg kg ⁻¹	576 ± 133	139 ± 64
50 mg kg ⁻¹	1514 ± 171	416 ± 85

All values are mean ± s.e.mean (n); ng g⁻¹ wet wt. tissue.

During the chronic treatment the values were of similar magnitude to those found 8 h after the acute injections, but lower than those found 2 h after injection.

Whole brain ethanol concentrations

Table 3 shows the whole brain concentrations of ethanol in groups of TO mice 5 h after withdrawal from ethanol treatment and in C57 mice 2 h after withdrawal. There was no significant difference between the concentrations following administration of ethanol alone and ethanol plus nitrendipine, in either strain, *P* > 0.1.

Discussion

Concurrent chronic administration of the dihydropyridine calcium channel antagonists, nitrendipine, nimodipine or PN 200-110, prevented the occurrence of the ethanol withdrawal syndrome. This effect was seen when 24 h was left between the removal of nitrendipine or the last injections of the chronic treatments and was still evident when a 48 h period was given between the last injections of nitrendipine and the withdrawal testing. The chronic treatment did not affect seizure thresholds to bicuculline in mice that were not given ethanol, indicating that it was not having a general, nonselective, anticonvulsant action.

We have found previously that administration of calcium channel antagonists acutely at the beginning of withdrawal from ethanol provided protection from the ethanol with-

drawal syndrome (Little *et al.*, 1986; Littleton *et al.*, 1990). However, the effects of the dihydropyridines described in the present results did not appear to be due to such acute actions. The brain nitrendipine concentrations at the time of withdrawal were longer than those in the CNS following acute administration of 10 mg kg⁻¹ nitrendipine. The 10 mg kg⁻¹ dose, given acutely on withdrawal, was ineffective, although 50 mg kg⁻¹ provided protection (Littleton *et al.*, 1990). When nitrendipine was given for the last 2 days only of the 2 week ethanol administration period, there was no effect on the withdrawal syndrome, suggesting that it was necessary to give the dihydropyridine for a certain length of time before the effects are seen. Two weeks administration was effective in both strains of mice.

The action of nitrendipine, given chronically, in preventing the ethanol withdrawal syndrome, parallels the effect of this compound in preventing development of tolerance to ethanol (Little & Dolin, 1987; Wu *et al.*, 1987; Dolin & Little, 1989). In the latter study we demonstrated that concurrent injections of nitrendipine, 50 mg kg⁻¹ i.p. once daily, in rats prevented the tolerance to the ataxic actions of ethanol that were seen after two weeks ethanol administration. The 10 mg kg⁻¹ dose of nitrendipine was ineffective in that study. We also showed that such concurrent nitrendipine administration prevented the increase in the number of dihydropyridine-sensitive binding sites in the cerebral cortex found after the chronic ethanol administration.

The action of nitrendipine in the present study was seen in both C57 and TO strains of mice, when different methods of ethanol administration were used and occurred whether nitrendipine was injected or dissolved in the drinking fluid. The effect was dose-dependent, 10 mg kg⁻¹ nitrendipine having less action than 50 mg kg⁻¹ when tested in the TO strain. Administration of ethanol to C57 mice for shorter periods than 12 weeks (for example, 5 weeks; unpublished observations) causes a pronounced withdrawal syndrome on cessation of treatment. The prevention of such a syndrome, despite the long period of ethanol administration, therefore suggests that 2 weeks' administration of nitrendipine reversed the changes in the CNS responsible for withdrawal hyperexcitability.

The action of nitrendipine was to decrease the ratings of convulsive behaviour. These measurements were made on a continuous rating scale, rather than being separated into tremor and convulsions, as it was apparent from the behaviour that there was a continuous gradation of severity, with no clear separation of the two signs (Littleton *et al.*, 1990). The effects of nitrendipine were not due to alterations in central ethanol concentrations or to differences in ethanol intake. We have previously shown that nitrendipine does not alter brain ethanol concentrations when given concurrently (Dolin, 1988, data quoted in Dolin & Little, 1989).

The doses of dihydropyridines used in this study were similar to those which have been demonstrated to be required for other actions on the central nervous system. Supervilai & Karobath (1984) showed that 10 mg kg⁻¹ of PN 200-110, i.p., was required to cause full displacement of *in vivo* dihydropyridine binding from rat brain. The doses of nitrendipine were the same as those found to prevent the development of tolerance to ethanol (Dolin & Little, 1989) and slightly lower than those required to prevent ethanol withdrawal when given acutely (Little *et al.*, 1986).

It is now recognised that there is a difference between the concentrations required for effects on central neurones and those effective on smooth muscle. This is thought to be due to the fact that dihydropyridines bind selectively to the inactivated form of the receptor (Bean, 1984). The effective concentrations are therefore dependent on the time the channels in different tissues spend in the inactivated state. One factor involved in this tissue selectivity could be the generally more hyperpolarized nature of neurones compared to smooth muscle cells, since the inactivation of the dihydropyridine-sensitive calcium channel involves membrane depolarization.

Table 3 Whole brain concentrations of ethanol (mg 100 g⁻¹)

(i) TO strain, 5 h after withdrawal from ethanol (n)		
Chronic treatment:		
Ethanol + vehicle injections	16.6 ± 10.5 (6)	
Ethanol + nitrendipine, 50 mg kg ⁻¹	14.5 ± 5.9 (7)	
(ii) C57 strain, 2 h after withdrawal from ethanol (n)		
Chronic treatment:		
Ethanol alone	48.1 ± 21.2 (7)	
Ethanol + nitrendipine	41.8 ± 12.8 (9)	

P > 0.1, comparisons between ethanol alone and ethanol plus nitrendipine, for each set of results (Student's *t* test).

We suggest that the selective action of nitrendipine described in the present results was, at least in part, due to an effect on the adaptive response of dihydropyridine-sensitive calcium channels to the chronic ethanol treatment. The measurements of dihydropyridine binding (Table 1) show that the increase in dihydropyridine receptor number caused by the ethanol was decreased by the concurrent nitrendipine treatment, the same effect being seen in both sets of experiments. This follows a similar pattern to that seen in our study on the effects of nitrendipine on ethanol tolerance (Dolin & Little, 1989) and to that found by Brennan & Littleton (1990) in a cultured cell line grown in ethanol.

We have demonstrated by electrophysiological studies (Whittington & Little, 1989a,b; Whittington & Little, 1991b) that nitrendipine prevented the evidence of neuronal hyperexcitability seen in isolated hippocampal slices following chronic ethanol administration *in vivo*. This action of nitrendipine was seen whether the compound was added to the perfusion medium during the electrophysiological recording or given to the animals during the chronic ethanol treatment, as in the present study. This shows that the behaviour seen after ethanol and nitrendipine chronic treatments was likely to be

due to an action on central neurones. Prevention of withdrawal-induced hyperexcitability in the hippocampal slice by addition of the dihydropyridine calcium channel antagonist, PN 200-110, to the bathing medium showed the same stereospecificity as the actions of this compound on calcium channels (Whittington & Little, 1990; 1991a).

The results provide evidence that the ethanol withdrawal syndrome may involve increases in central dihydropyridine-sensitive calcium channels. We suggest that the effect was due to an adaptive response to the long-term presence of the dihydropyridine, involving prevention, or reversal, of the ethanol-induced increase in dihydropyridine-sensitive calcium channels. Nitrendipine did not cause any withdrawal syndrome when given chronically in the absence of ethanol. This action of dihydropyridines may therefore provide a new approach to ethanol dependence, that could alter the neuronal adaptations to chronic ethanol intake, rather than simply substituting for ethanol, as do the drugs in current use.

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