CORRELATION BETWEEN INCREASES IN DIHYDROPYRIDINE BINDING 
IN VIVO AND BEHAVIOURAL SIGNS OF ETHANOL WITHDRAWAL IN 
MICE

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Abstract — Increased ligand binding to dihydropyridine receptors in the central nervous system after chronic ethanol consumption is thought to contribute to the withdrawal syndrome. Previous studies demonstrated such changes when the binding was measured in vitro, which, as the receptors are voltage-sensitive, may not accurately reflect the binding in vivo. In the present study, dihydropyridine binding was measured in vivo in mice, after intravenous administration of the radioligand. The aim was to determine whether there was any correlation between such binding and measurements of behavioural hyperexcitability at different times during the withdrawal phase and after two different methods of alcohol administration. Measurements were made of the binding in vivo of [3H]nitrendipine, at intervals after withdrawal from chronic ethanol administration, and of the severity of withdrawal as measured by response to gentle handling. An increase in the in vivo binding to [3H]nitrendipine was seen after cessation of chronic ethanol consumption by liquid diet. The binding was significantly increased at 4 h, when the behavioural changes were maximal, but not immediately after withdrawal, when the responses to handling were unchanged. By 24 h after cessation of the ethanol treatment, no differences in the binding were found, compared with control values; at this time the withdrawal hyperexcitability had ceased. When alcohol was given chronically by inhalation, the in vivo dihydropyridine binding was increased at 3 h from withdrawal of the ethanol, the time of maximal behavioural hyperexcitability, but no change was seen 30 min after withdrawal, when no changes in the ratings of behaviour were found. There was a significant positive correlation in individual mice between the ratings of handling-induced behaviour at the 3 h interval and the amount of in vivo binding. These data support the hypothesis that the sites labelled by [3H]nitrendipine play an important role in withdrawal hyperexcitability.

INTRODUCTION

Previous evidence has suggested that dihydropyridine-sensitive sites may be involved in the behavioural and other changes that occur on withdrawal from alcohol administration. The sites at which dihydropyridine compounds bind in the central nervous system are considered to represent the ‘L’ subtype of calcium channel. The involvement of these channels in the ethanol withdrawal behavioural syndrome was originally suggested by the observation that administration of the dihydropyridine calcium channel antagonists, nitrendipine, nimodipine and isradipine significantly decreased the withdrawal convulsive behaviour in both rats and mice (Dolin et al., 1987; Littleton et al., 1990). The calcium channel activator Bay K 8644 prevented the action of nimodipine in blocking the ethanol withdrawal syndrome (Littleton et al., 1990), and hyperexcitability in hippocampal slices prepared after chronic ethanol administration in vivo was found to be prevented by dihydropyridine calcium channel antagonists (Whittington et al., 1991; Bailey et al., 1998).

The density of dihydropyridine binding, measured in vitro in rat brain after in vivo administration of ethanol, was found to be increased after prolonged ethanol treatment (Dolin et al., 1987). The effects of dihydropyridine calcium channel antagonists on phosphatidyl inositol turnover and neurotransmitter release were also increased after the chronic ethanol administration (Dolin et al., 1987). Subsequent binding studies have shown that the in vitro dihydropyridine binding is increased 3–4 days after the beginning of chronic ethanol administration and that the increase was

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no longer present at 24 h after cessation of the ethanol treatment (Guppy et al., 1995). These studies, however, all measured dihydropyridine binding in vitro in brain homogenates. The binding in vivo may differ from that measured in vitro, as dihydropyridine binding is known to be voltage-sensitive and the binding is influenced by the local calcium concentration (Thayer et al., 1986; Guppy and Littleton, 1994). Differences in the level of excitability of neurones may therefore affect the receptors in vivo, while the binding measured in vitro is in homogenized, and therefore completely depolarized, cells. It is known that, during the ethanol withdrawal syndrome, neurones become hyperexcitable (Whittington and Little, 1990, 1993).

The aim of the present study was to compare the dihydropyridine binding, measured after administration of a radioligand to conscious animals ('in vivo' binding), and the severity of the withdrawal syndrome, as measured by responses to gentle handling. Two methods of administration of ethanol were used, liquid diet and inhalation, to determine whether the results were dependent on the method of administration, and therefore pattern, of ethanol intake. Comparison was made between the time-course of the changes in behaviour and dihydropyridine binding and the ratings of withdrawal severity and, in the second experiment, the in vivo binding in individual mice.

METHODS

Male mice of the TO strain were used throughout. They were kept in a 12 h light/12 h dark cycle with access ad libitum to tap water and laboratory rodent chow (CRM). The weight range was 30–40 g, with no more than a 5 g range in any single experiment.

In the first experiment, ethanol was administered by the liquid diet method, in which a complete diet is provided containing ethanol. The mice drink this diet primarily during the night, but some consumption takes place during daytime. In the second method, ethanol was administered by inhalation, which provides a constant 24 h equilibrium between the concentration of ethanol in the blood, and therefore brain, and that in the inhaled air. There were no differences in the weights of the ethanol-treated and control mice at the end of the treatment periods for either method of ethanol administration.

In vivo dihydropyridine binding

The methods were those of Shoemaker et al. (1983) and Supavilai and Karobath (1984), as described previously (Watson and Little, 1994). An injection of 4 μCi of [3H]nitrendipine (specific activity 71.2 Ci/mmol) in a volume of 0.1 ml was given into the tail vein. The mice were killed by cervical dislocation, 15 min after injection of radiolabel. The brain was then removed and homogenized at a concentration of 20 mg/ml in ice-cold Tris-Cl buffer (50 mM, at pH 7.4). One ml of the homogenate was placed in a scintillation vial and label count was used as a measure of the total activity in each homogenate, to provide a correction for differences in the amount of ligand given to each mouse (owing to differences in body weight, cerebral blood flow or metabolism). Another 1 ml of each homogenate was then filtered through Whatman GF/B (2.5 cm) filters using a filtration manifold system, and the filters were washed quickly by 2 × 5 ml of ice-cold buffer (Tris-Cl, 50 mM, pH 7.4). The activity count provided a measure of the activity bound to the membrane fragments (particulate binding). Each measurement was performed in triplicate. Four ml of scintillation fluid (Hi-ionic Fluor, Packard) were added to each vial and the samples counted in a scintillation counter (Packard, Tricarb). Non-specific binding was assessed in the present study by incubating a further 1 ml sample of each homogenate with 10 mM nimodipine at room temperature. This was then filtered and counted as above.

In our previous work (Watson et al., 1994), in addition to the above method of determining non-specific binding, we also counted the amount of radiolabel in the receptor-poor area of the brain stem and surrounding white matter. The results obtained using these two methods were not significantly different. In the current study, the non-specific binding was of the order of 22% and there were no significant differences in this between treatment groups [e.g. for experiment 2: $F(2,32) = 1.08; P = 0.35$].

The specific binding was expressed as a percentage of the total activity in the brain homogenate, i.e. the particulate counts minus the non-specific counts, divided by the total counts in
1 ml of homogenate. The results were expressed as means and standard errors (SEM) for each treatment group.

Production of dependence by the liquid diet method

Ethanol was administered in a liquid diet schedule (Dyets, Pennsylvania; Lieber and DeCarli, 1989). All mice received the control diet for an initial 2 day period. Ethanol-treated mice then received a diet containing 3.5% (v/v) ethanol for 2 days, followed by a diet containing 7% (v/v) ethanol for a further 5 days. Control groups were pair-fed a control diet, balanced isocalorifically to match the ethanol-containing diet (Lieber and DeCarli, 1989). The amount of alcohol consumed by the mice was 18–22 g/kg/24 h.

Production of dependence by the inhalation method

Mice were made physically dependent on ethanol by a 14-day period of inhalation of ethanol vapour at an ethanol concentration of 6–10 mg/l in normal air, in an inhalation chamber (Green et al., 1990). Control animals were kept under identical conditions, in the absence of ethanol.

Handling-induced behaviour

Ratings of handling-induced behaviour were assessed after withdrawal from ethanol, by the same experimenter each time, who was blind to the prior treatment. This method, widely used in the study of ethanol withdrawal, follows that of Goldstein and Pal (1971), modified slightly in our laboratory (Green et al., 1990; Watson et al., 1994). Each mouse was lifted gently by the tail and held for 3 s at 10 cm below an ‘Anglepoise lamp’ with a 60 W bulb. The animal was gently rotated and its ensuing behaviour rated on a scale of 0–5 according to the criteria in Table 1.

Experimental design

Measurements of the behaviour during withdrawal were made in the first (liquid diet) experiment, at 1 h intervals for 12 h, in mice that were not used for the binding measurements (n = 12), to determine the time of maximal response. Measurement of the binding to the dihydropyridine, [3H]nitrendipine, was made in separate groups of mice (n = 10) after intravenous injection of this ligand in vivo, immediately after withdrawal of ethanol (0 h time interval), at 4 h after withdrawal, when the handling response was maximal, and at 24 h after withdrawal, when the behaviour had returned to normal.

In the second experiment, alcohol was given by inhalation. In a separate group of animals (n = 12) the whole time-course of the withdrawal hyperexcitability was monitored, at 1 h intervals for 12 h. From these results, a time of 3 h after removal of mice (n = 8) from the ethanol inhalation chamber was chosen as being the time at which the withdrawal hyperexcitability was maximal. Measurement of the in vivo binding was then carried out in separate groups of mice at 30 min and 3 h after cessation of ethanol treatment. In the same animals (n = 8) in which the binding was measured at the 3 h time interval, the responses to gentle handling were rated, immediately prior to the injection of the ligand. This enabled direct comparison to be made between the behaviour and the binding in individual mice.

Statistical analysis

The results of the handling response ratings were compared by non-parametric two-way analysis of variance, designed for repeat measures on the same animal (Meddis, 1984). Differences in the in vivo binding were compared by Student’s
The correlation in the second experiment, between the handling responses and the \textit{in vivo} binding, was performed using Spearman’s rank correlation coefficient. The total activity in homogenates, and non-specific binding, for all treatment groups were compared by one-way analysis of variance.

**RESULTS**

**Administration of ethanol by liquid diet**

The ratings of handling-induced behaviour (Fig. 1) demonstrated significant increases in the behavioural responses between 2 and 8 h from cessation of alcohol consumption. The maximal response to handling occurred between 2 and 5 h from cessation of alcohol intake, with the most consistent responses at the 4 h time interval. This time was therefore chosen for the \textit{in vivo} binding measurements. By 24 h after cessation of ethanol consumption, the behavioural signs of withdrawal had completely disappeared (data not illustrated).

The results of the \textit{in vivo} dihydropyridine binding are illustrated in Fig. 2. There was significantly higher binding at 4 h into the withdrawal period ($P < 0.05$), compared with the binding at 0 h. There were no significant differences between the binding at 0 h (immediately upon withdrawal) or the 24 h withdrawal point, compared with control values.

**Administration of ethanol by inhalation**

The ratings of responses to handling, illustrated in Fig. 1, showed a significant increase between 1 and 8 h after removal of the animals from the inhalation chamber. The \textit{in vivo} binding (Fig. 3) showed a significant increase in the dihydropyridine binding at 3 h after withdrawal from the ethanol chambers ($P < 0.05$), compared with the values at 30 min and those of controls. As we reported in our previous studies (Watson and Little, 1994), there was no difference between the groups in the total binding activity in each homogenate [$F(2,32) = 0.77; P = 0.47$].

Figure 4 shows the behavioural ratings in individual mice, at the 3 h time interval, plotted against the \textit{in vivo} binding to [$^{3}H$]nitrendipine in each animal. There was a significant correlation ($P < 0.05$) between the severity of the withdrawal behaviour and the dihydropyridine binding.

**DISCUSSION**

A positive correlation was seen between the binding to the dihydropyridine ligand, administered \textit{in vivo}, and the severity of the withdrawal behaviour.

The results showed that measurement of dihydropyridine binding after injections of labelled ligand \textit{in vivo} was in agreement with the
results from the earlier in vitro binding studies. Littleton and colleagues demonstrated that the characteristics of dihydropyridine receptors measured in vitro after chronic ethanol treatment in vivo were very similar to those studied in controls (Guppy and Littleton, 1994; Guppy et al., 1995). This group also demonstrated that up-regulation of dihydropyridine binding in cultured cells was prevented by an mRNA and protein synthesis inhibitor (Harper et al., 1989), indicating that the increase in binding involved the synthesis of new protein. The results are also in agreement with the early work using cultured cells, in which prolonged alcohol treatment caused an up-regulation of dihydropyridine binding (Messing et al., 1986).

One difference from previous work was that, in the present study, the binding was not increased at the beginning of withdrawal, but only when the signs of behavioural hyperexcitability were apparent. The measures of receptor binding in vivo, however, were made in the intact preparation, when the neurones would be in the ‘real life’ state of polarization, rather than the totally depolarized state in which homogenate binding is measured. At the time of withdrawal, there would still be alcohol in the brains of the mice. The study of Watson and Little (1995) showed that measurable amounts of ethanol were present in the brain up to 2 h into the withdrawal period, after the liquid diet treatment schedule used in the current study. The presence of ethanol would affect the state of depolarization and so may affect calcium channel binding.

Previous studies have demonstrated that the dihydropyridine binding measured in vivo is displaced by dihydropyridine calcium channel antagonists administered in vivo (Shoemaker et al., 1983; Supavilai and Karobath, 1984; Watson et al., 1994). Administration of unlabelled dihydropyridine decreased specific binding in vivo, but did not alter the total amount of radioactivity in the tissues. Specific dihydropyridine binding is increased by the inactivation of the channels that
follows depolarization (Bean, 1984; Triggle et al., 1989). The increase in binding in vivo therefore could be due to increased numbers of receptors or to increased proportion of inactivated channels. The earlier results from in vivo binding, carried out on homogenized and therefore depolarized tissue, indicated that the former is the case (Dolin et al., 1987; Guppy et al., 1995). The prevention of the behavioural signs of withdrawal by dihydropyridine calcium antagonists (Dolin et al., 1987; Littleton et al., 1990) suggests that the increased binding is a factor contributing to the withdrawal hyperexcitability, rather than a result of the withdrawal.

Our previous work, however, has demonstrated that the extent of displacement of in vivo binding does not exactly parallel the behavioural effects of the drugs in protecting against the ethanol-withdrawal hyperexcitability (Watson et al., 1994). Felodipine, a dihydropyridine calcium channel antagonist, caused displacement of in vivo binding, but gave no protection against the behavioural effects of withdrawal. It is possible, however, that effects of felodipine, exerted at other sites, counteract a potential protective action due to the interaction of the compound with dihydropyridine receptors.

Measurements of binding in vivo have been used to indicate the extent of occupation of receptors by endogenous compounds. For the dihydropyridine receptor, it is not known if there is an endogenous compound(s) which acts at these sites in vivo. Some candidates have been suggested for such a ligand (Baydoun et al., 1988; Hanbauer et al., 1988), but little evidence is available to support these. It may be that there is no endogenous ligand for these sites, and if one exists it could have actions similar to the dihydropyridine antagonists, decreasing channel conductance, or might act in the same way as calcium channel agonist drugs, such as Bay K 8644, increasing channel conductance. No definite conclusions can therefore be drawn about the involvement of such a ligand in the ethanol withdrawal syndrome, although the existence of such a compound remains a possibility. It is likely that an endogenous ligand would be removed during the tissue preparation in the in vivo binding studies [although for some receptors, e.g. glucocorticoid binding (Spencer et al., 1990), endogenous ligands are not removed during such procedures]. The close parallels, therefore, between the changes in binding measured in vitro and in vivo suggest that any endogenous ligand is unlikely to play an important role.

Our previous studies have provided considerable information about the time-course and duration of the withdrawal syndrome produced by cessation of the above two methods of ethanol administration. The patterns of ethanol withdrawal after the inhalation and liquid diet methods of alcohol administration are very similar. The withdrawal signs after ethanol inhalation are usually slightly more severe and are seen slightly earlier than those after liquid diet withdrawal. This difference is likely to be due to the amount of alcohol taken in by the mice in each case, and the patterns of blood ethanol achieved by each method. The binding results showed clearly that the up-regulation of dihydropyridine binding does not depend on the method used for chronic administration of ethanol, or on the pattern of its intake. The percentage specific binding in the brains of control animals was not significantly
different in the two experiments, although the mean values were slightly higher in the study in which alcohol was given by inhalation. It is possible that this minor variation was due to the experiments being carried out at different times, several months apart.

In conclusion, dihydropyridine receptor binding measured in vivo shows a clear correlation with behavioural signs of hyperexcitability due to withdrawal from chronic ethanol administration. The in vivo binding changes paralleled those measured in vitro after chronic ethanol treatment which demonstrated that up-regulation of dihydropyridine-sensitive sites does take place in vivo during chronic ethanol treatment.

REFERENCES


