

**Final Report on MRC Programme Grant G 9806260****Sensitisation to repeated withdrawal from alcohol and benzodiazepines.****DN Stephens, T Duka, M. O'Shea****(September 1999 - August, 2004)****Background**

It has been known for some years that alcoholics undergoing detoxification undergo a risk of seizures, and that this risk increases with increased numbers of detoxifications. Although clinical studies are not able to dissociate numbers of detoxifications from lifetime alcohol consumption, or duration of alcohol abuse, rodent experiments controlled for total intake have demonstrated convincingly that increased seizure sensitivity following repeated withdrawal in alcohol-dependent animals depends upon the number of previous withdrawals. This phenomenon has been suggested to result from withdrawal initiating a process akin to epileptic kindling, and consonant with that idea, repeated experience of alcohol withdrawal pre-sensitises rats to electrical kindling of the inferior colliculus and amygdala (but not hippocampus), and amygdala kindling facilitates the development of withdrawal-induced convulsions. Although increase in severity of seizure-related phenomena has been well documented in both the clinic, and in the animal laboratory, little information has previously been available on other sequelae of repeated withdrawal. We have carried out a series of experiments investigating affective and cognitive consequences of repeated alcohol withdrawal in rodents, and in alcoholic patients, and social drinkers, and have begun to investigate the underlying neurobiological events initiated by withdrawal.

**Patient Studies in Alcoholics with multiple Detoxifications**

In our initial studies with patients, we chose to investigate mildly dependent alcoholic inpatients more than one week into detoxification in a private clinic. This population was chosen for study, partly because it represented an early stage of alcohol dependency, but also because the patients for the most part had no pharmacological treatment during their detoxification, which might have masked any effects of repeated detoxification. Compared to a population of social drinkers, the patients revealed heightened trait anxiety, and exaggerated feelings of anger anxiety, depression and confusion (but also friendliness); they also reported higher craving ratings, and made more errors in a version of the Stroop test using alcohol-related words as distractors. When the alcoholic population was divided on the basis of the number of previous withdrawals, patients with more than 2 previous detoxifications showed higher anger scores, and made more errors in the Stroop test using negative alcohol-related words (e.g. hangover; vomit) than patients who had 2 or fewer detoxifications, indicating heightened attention to such words. These differences remained when the correlates alcohol consumption and degree of dependency were introduced into the analysis as covariates<sup>1</sup>. Importantly, no differences were found in a colour Stroop task used as a control task<sup>2</sup>.

We have also investigated the same patients using tasks sensitive to dysfunction of prefrontal areas, some of which have previously been shown to be sensitive to chronic alcohol use. The tasks applied were two maze tasks from the WISC-III, and the vigilance task for adults and the delay task from the Gordon Diagnostic System. The alcoholic patients took more time to complete maze 1 and made more errors in both mazes. They also made more commission errors and gave fewer correct answers in the vigilance task. Patients with 2 or more previous experiences of medically supervised detoxifications were more impaired than patients with a single, or no previous experience of detoxification in the maze 1 and in the vigilance task; they were also more impaired in the delay task. Repeated experience of withdrawal from alcohol is thus associated with impaired cognitive function as assessed using tasks sensitive to frontal lobe deficits<sup>2</sup>.

The animal studies indicate that repeated alcohol withdrawal sensitises amygdala neurotransmission (see below). In order to approach whether amygdala function was altered in our patient population, we made use of the knowledge that the amygdala is intimately involved in encoding of emotions, especially those related to fear. In human subjects, the ability to recognise a facial expression indicating fear involves activation of the amygdala, suggesting a neuropsychological test for altered amygdala function in repeatedly-withdrawn alcoholics. Recognition of the emotional content of facial expressions is a central feature of emotional and social behaviour and previous studies have found that alcoholics are impaired in this skill when presented with single emotions of differing intensities. Fourteen alcoholic inpatients were compared with 14 age and sex matched social drinking controls in their rating of each of six emotional facial expressions (happiness, surprise, fear, sadness, disgust and anger) present in morphed pictures portraying a mix of two of those emotions. The alcoholic group showed

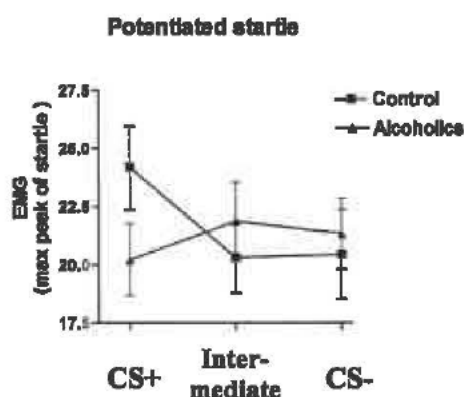
enhanced fear responses to all of the pictures compared to the controls and showed a different pattern of responding on anger and disgust. There were no differences between groups on decoding of sad, happy and surprised expressions. Importantly, the enhanced fear recognition found in the alcoholic group was related to the number of previous detoxifications. These results provide initial evidence from mild to moderately dependent patients that impairment in facial expression recognition by alcoholic patients may be attributable to altered amygdala function following repeated detoxification<sup>3</sup>. Studies investigating a more severely dependent group, which has undergone a higher number of detoxifications, have recently been completed. Preliminary evaluation of the data has shown that severely dependent alcoholics (in-patients with history of fits and/or severe blackouts) do not differ from the less severely dependent alcoholics (out-patients) with respect to trait or state anxiety; in-patients were, however, more impaired in tasks of executive cognitive function and in the emotional facial expression recognition of anger.

## Binge Drinking

Our general hypothesis posits that the brain adapts to the inhibitory effects of alcohol by increasing its excitatory tone, possibly by up-regulating glutamatergic transmission, and that withdrawal of the alcohol leaves untrammelled expression of this increased excitability, resulting in activity-induced plasticity. We suggest that binge drinking, in which the drinker regularly, but intermittently, consumes large quantities of alcohol, may have similar consequences. We have therefore begun to investigate behavioural changes resulting from a binge pattern of drinking.

We evaluated a method for establishing patterns of drinking among young alcohol users, and developed criteria for their classification into binge drinking, and non-binge drinking patterns<sup>4</sup>. Duka was invited by the American National Institute of Alcohol Abuse and Alcoholism (NIAAA) to present our approach as a new tool for the diagnosis of binge drinking. Using this method, we have identified binge-drinking patterns of behaviour among social drinkers that do not merely reflect the quantity of alcohol consumed. Among a group of individuals with light to heavy use of alcohol (3 to 120 units per week;  $n=70$ )<sup>5</sup>, binge drinkers gave lower "elated" scores than non-binge drinkers with similar weekly intakes, had less positive mood, and were higher in the temperament trait "novelty seeking" (previously been linked with early onset alcoholism<sup>6</sup>); they also had more positive outcome expectancies from alcohol drinking than non-binge drinkers. In cognitive tasks, binge drinkers had faster reaction times in a matching to sample visual searching task (consistent with increased impulsivity) and performed worse in a spatial working memory task than non-binge drinkers, although the latter effect was gender-dependent (found in female subjects). When alcohol was administered to another group of individuals classified as binge-drinkers and non-binge drinkers ( $n=50$ ; 12 to 45 units per week) no differential impairing effect of alcohol was found, but binge drinkers were impaired compared to non-binge drinkers in a spatial working memory task and in a pattern recognition task, suggesting again that binge drinking may be associated with impaired cognitive function<sup>7</sup>. The amount of alcohol used per week by the individuals did not predict any of the impairments seen in binge drinkers, though the age at which individuals started to drink was an additional predictor of impaired cognitive abilities. We do not yet have sufficient data from binge drinkers to conclude that they resemble alcoholics who have undergone repeated episodes of detoxification, but the cognitive deficits seen in both conditions may suggest some similarities. Particularly interesting is our observation that binge drinkers show deficits in fear conditioning<sup>8</sup> analogous to the deficits we have already reported for rats which have undergone several episodes of repeated withdrawal<sup>9,10</sup>, and which we have now confirmed for alcoholic patients (Fig 1; in preparation).

None of our studies on alcohol related positive reinforcement measurements (e.g: craving for alcohol<sup>1</sup>; priming by alcohol<sup>11</sup>; or alcohol effects on emotional memories<sup>12</sup> found differences between the different subgroups of alcoholic patients or between binge drinkers and non-binge drinkers.



**Fig 1:** Potentiated startle responses of 14 alcoholic patients compared to 14 social drinkers, trained in an auditory discrimination procedure using 3 tones of 65dB intensity, but different frequencies (low: 900Hz; intermediate: 1300Hz; and high: 1700Hz). The CS+ (low or high, counterbalanced across subjects) predicted an aversive white noise of 97dB. The aversive CS+ potentiated startle in control subjects when compared to either the intermediate, or CS- stimulus. Alcoholic patients did not show potentiated startle to the CS+.



## Animal Studies of Repeated Withdrawal from Alcohol

Animal studies have sought to model some of these findings, and to investigate them in a more controlled fashion.

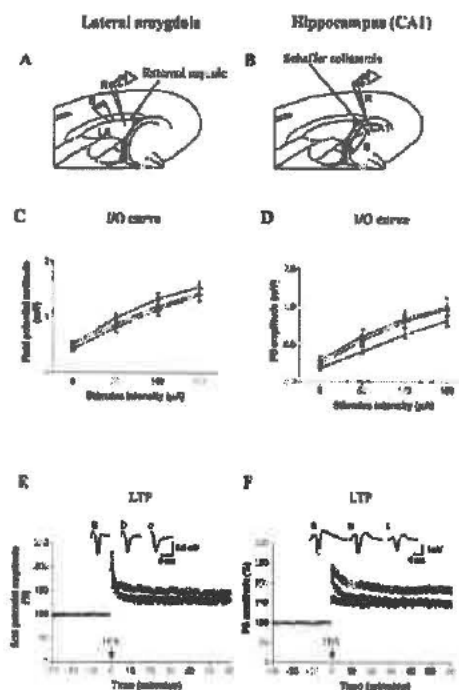
Recent reports in the literature<sup>13</sup> indicates that rat anxiety assessed in a social interaction test is increased during acute withdrawal and that the magnitude of this effect is increased following repeated withdrawal. We have observed a similar phenomenon in mice in two simple models of anxiety, the plus maze and open field tests (in preparation). However, both the social interaction test, and the plus maze and open field tests of anxiety are strongly influenced by changes in activity, and we are cautious in interpreting these data as indicating increased withdrawal-induced anxiety following repeated withdrawal experience. One reason for such caution is that measurement of plasma corticosterone levels 8h following withdrawal from our alcohol regimen shows marked increases during the first experience of withdrawal, suggesting withdrawal is stressful for the rats; however, following repeated withdrawal experience, withdrawal does not induce elevated corticosterone levels, suggesting it may no longer be aversive (Le Merrer and Stephens, in preparation).

In order to understand a possible role for facilitated anxiety following repeated withdrawal in the long-term consequences of alcohol dependence, it is important to examine behaviour some time after acute withdrawal symptoms have subsided. We have therefore concentrated our work on behavioural events at least two, and sometimes many more weeks following alcohol treatment and withdrawals. At this time, rats which have undergone 3 repeated experiences of withdrawal show impaired fear conditioning compared to both control rats, and rats which have consumed the same amount of alcohol (approximately 15g/kg/day for 24 days, giving rise to blood alcohol concentrations of about 100 mg/dl), but have undergone only a single withdrawal<sup>9</sup>. This observation was unexpected, not only because it is widely accepted that alcoholism leads to increased anxiety, and increased frequency of panic attacks even in abstaining alcoholics following multiple experience of withdrawals<sup>14</sup>, but also because we have found that electrical kindling of the amygdala, or chemical kindling with the GABA antagonist, pentylenetetrazole, both of which have been suggested to cross-sensitise to repeated withdrawal in convulsant activity<sup>9,15</sup>, in our hands *facilitate* the acquisition of conditioned fear<sup>16</sup>. Expression of conditioned fear, acquired prior to induction of alcohol dependence, was unaffected by repeated withdrawal, though both extinction of that conditioned fear, and learning of a new association was impaired<sup>17</sup>. As mentioned above, we have subsequently found an analogous deficit in fear conditioning in binge drinkers, illustrating the utility of being able to test readily in humans, hypotheses derived from animal experiments.

Fear conditioning depends upon associations being made between an aversive event (in our case, mild footshock), and an environmental cue (in our case a brief tone, or a light), and such associations are known to depend upon plasticity in the lateral part of the amygdala<sup>18</sup>, so that it is conceivable that as a result of repeated withdrawal experience, the amygdala circuits may not be capable of forming associations necessary for fear conditioning. In keeping with impaired conditioning, a tone previously paired with footshock, which induced the expression of *c-fos*, a marker of neuronal activity, in amygdala and other limbic areas in control animals, did so to a lesser extent in rats which had previously experienced repeated withdrawals<sup>8</sup>.

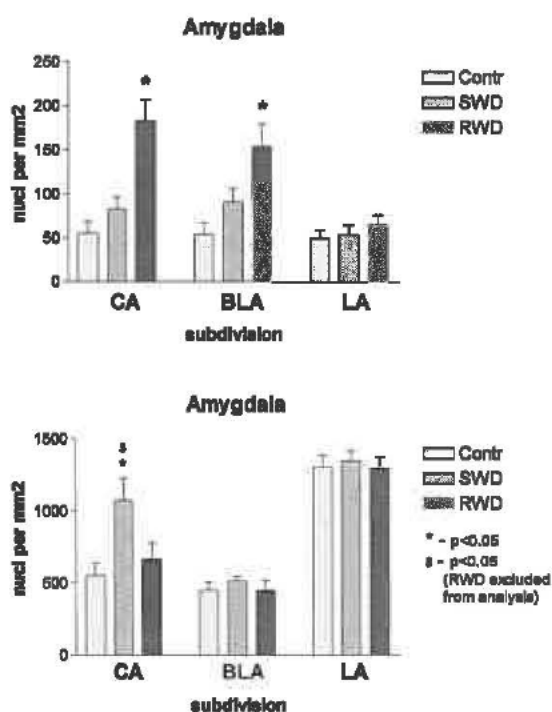
The increased seizure-sensitivity following repeated withdrawal, but impaired conditioning, may be reconciled by postulating that heightened neuronal activity during acute alcohol withdrawal leads to facilitation of synaptic transmission in amygdala, leading eventually to saturation. Such saturated synapses would facilitate seizure spread, but result in reduced capacity for further plasticity necessary for learning. In keeping with that hypothesis, in electrophysiological experiments carried out in collaboration with [REDACTED]

[REDACTED] repeated withdrawal rats showed reduced long term potentiation in lateral amygdala following theta stimulation of external capsule afferents (fig 1). Such changes were not limited to the amygdala, and similar impaired long term potentiation was also seen in recordings from hippocampus CA1 neurones activated by stimulation of the Schaffer collaterals<sup>8</sup>.



**Fig 2:** The effects of single (SWD -▲-;  $n = 8$  rats) and repeated withdrawal (RWD -■-;  $n = 8$  rats) on excitability (input/output curves (C, D) and long-term potentiation (E,F) in comparison to control brain slices (-○-;  $n = 6$  rats). A, B: Schematic representation of horizontal brain slices used in electrophysiological experiments showing the location of the recording electrode (R) in the lateral nucleus of the amygdala (A) and in the pyramidal layer of the CA1 region of the hippocampus (B), respectively. Bipolar stimulation electrodes (S) were used to stimulate either external capsule fibres (A) or Schaffer collaterals (B). The input/output (I/O) response curve (C, D) was constructed by varying the intensity ( $\mu A$ ) of single-pulse stimulation above threshold, and averaging 6 responses at each intensity. LTP in the LA and CA1 are shown in figures E and F respectively. Data points represent averaged amplitudes (mean  $\pm$  SEM) of field potentials (LA) and PS (CA1) normalized with respect to baseline values. Application of high frequency stimulation (HFS) and TBS occurred at time 0. Traces show representative examples of evoked field potentials in the LA and in the CA1 5 min before (broken line) and 60 min after HFS or TBS, respectively (a: controls; b: SWD; c: RWD). Note the significant difference in LTP in both brain structures between controls and SWD, and controls and RWD, respectively ( $p < 0.01$ ). SWD and RWD also significantly differed in CA1 ( $p < 0.01$ ). Number of brain slices used for the construction of the I/O curves and LTP was 12 to 27 in the lateral amygdala and 9 to 27 in the CA1.

We have also addressed the same question using histochemical techniques. Induction of the immediate early gene, *c-fos*, is frequently used as a measure of neuronal activity, whereas induction of a different immediate early gene, *zif-268* (also known as *Egr1*, *NGFI-A*, or *Krox24*), appears to parallel synaptic plasticity<sup>19,20</sup>. Withdrawal from our chronic alcohol regimen results, 8h later, in increased expression of *c-fos* in several brain areas, including central and basolateral nuclei of the amygdala, consistent with heightened neuronal activity in these areas; following repeated withdrawal experience, *c-fos* expression was further increased, consistent with further heightened neuronal activity (fig 2). *Zif-268* expression showed a rather different pattern; although *zif-268* expression was increased in central amygdala during the first withdrawal, we did not find such increases if the animals had experienced previous withdrawals, suggesting that synapses were no longer undergoing plasticity in these areas, consistent with our hypothesis of synaptic saturation (Borlikova and Stephens, in preparation).



**Fig 3:** Expression of *c-fos* (upper figure) and *zif268* (lower figure) immunoreactivity in amygdala following control, single withdrawal or repeated withdrawal treatments. \* Indicates different from control group,  $p < 0.05$ . CA: central amygdala nucleus; BLA: basolateral amygdala; LA lateral amygdala.

This model makes an interesting prediction. Conditioning requires strengthening of specific synaptic connections between CS pathways and CR pathways; by strengthening synapses unselectively, RWD might result in inappropriate stimuli gaining access to a previously conditioned CR. To test this prediction, rats were trained in a conditioned fear paradigm with different tone stimuli as CS+ and CS-. Following chronic ethanol treatment, RWD rats showed generalisation of conditioned fear from the CS+ to both the former CS-, and an intermediate novel tone stimulus<sup>8</sup>. These observations suggest that alcohol withdrawal increases synaptic efficiency, with two consequences; inappropriate generalisation of previously learned fear stimuli, and impairment of future learning. In principle, such a mechanism may account, not only for our observations on fear conditioning, but also for the alcoholic's inappropriately anxious response to non-threatening stimuli seen in panic attacks, and inappropriate recognition of fear in facial expressions. If the changes seen in amygdala and hippocampus occur in other parts of the brain, then more general cognitive disturbances might be expected. For example, in a test of negative patterning, although repeatedly withdrawn rats were able to learn a go—no-go task when food availability was signalled by either a light cue or a tone cue, they were impaired in learning that when the light and tone cues were presented together, they signalled non-availability of food (Borlikova and Stephens, in preparation).

This set of hypotheses, derived from close interactions between scientists involved in clinical and animal experimental studies, has generated a number of predictions that we are currently following. The animal studies predict that although repeated experience of withdrawal may exacerbate previously learned anxiety reactions, the same alcoholic subjects may learn about new threats less readily, and we have already confirmed that alcoholics (fig 1) and binge drinkers<sup>8</sup> show such deficits in fear conditioning.

Secondly, in addition to fear conditioning, appetitive conditioning also depends on plastic processes within the amygdala, raising the question whether the appetitive properties of alcohol may also be altered following repeated withdrawal. We have previously provided some evidence, from increased breaking points in progressive ratio schedules, that repeated withdrawal experience increases the extent to which rats are willing to work for ethanol<sup>21</sup>, and these findings have been confirmed by others. However, the rats perform poorly in responding for ethanol on progressive ratio schedules, possibly because the drug is sedative, and we have questioned the standard interpretation of elevated breaking points in terms of increased motivation<sup>22</sup>. For this reason, we have developed a new approach to study motivation for ethanol, in which rats are allowed to lever press to obtain 20 mins free access to a 10% ethanol solution, rather than being reinforced by small (usually 0.1ml) aliquots. Using this new schedule rats will emit up to 200 responses in a 1h session to obtain reinforcement. During a week's testing, we set the response requirement variably for each day, so that each animal will receive reinforcement after 16, 32 or 64 responses, and one day each week is run in extinction. On these extinction days, it is therefore possible to test animals drug-free. We are currently using this method, which we have named the Daily Variable Ratio (DVR) schedule, to study the effects of previous experience of chronic ethanol exposure, and single and repeated withdrawal on motivation for ethanol. Following alcohol treatments (either SWD or RWD) we find decreased lever pressing in extinction trials when the reinforcer is ethanol, but not when it is 3% sucrose, suggesting that several weeks following ethanol withdrawal experience, rats do not show heightened motivation to obtain ethanol solutions (unpublished). This observation is congruent with our failure to find evidence that repeated detoxifications affected alcohol craving measures in our patient studies, but seems at odds with claims from other laboratories that cycles of access to, and deprivation from ethanol, increases alcohol consumption (so-called alcohol deprivation effect). In collaboration with the pharmaceutical industry, we have also used the DVR method to study the ability of novel putative treatments of alcohol abuse to reduce motivation to drink. Drugs acting as inverse agonists at the benzodiazepine site of GABA<sub>A</sub> receptors containing  $\alpha 5$  subunits reduced the motivation to consume alcohol (in preparation) while the ability of non-specific inverse agonists to reduce lever pressing for alcohol was abolished in mice with targeted deletion of  $\alpha 5$  subunits (in preparation).

The amygdala sub-regions involved in appetitive conditioning have been well described<sup>23, 24</sup>, and given the evidence suggesting alterations of amygdala-dependent fear conditioning following repeated withdrawal, we have investigated aspects of appetitive conditioning following these treatments. In conditioned behaviours that are impaired by lesions of the basolateral amygdala (BLA), conditioned reinforcement and reinforcer devaluation, there was no effect of prior chronic ethanol treatment or withdrawal on acquisition or performance. However, in a task that is dependent upon the central nucleus of the amygdala (CA), Pavlovian-to-instrumental transfer, the SWD and RWD groups were significantly impaired. These results suggest that neuronal transmission in the CA, but not the BLA, may be affected by chronic ethanol treatment or ethanol withdrawal, but this impairment was not increased by repeated experience of ethanol withdrawal<sup>15, 25</sup>. We also found no deficits following repeated withdrawal in place preference conditioning to amphetamine<sup>15</sup>, a behaviour also disrupted by basolateral



amygdala lesions, following ethanol treatment. Given the evidence that fear conditioning was impaired by repeated withdrawal treatment, and that fear conditioning has been usually ascribed to associative mechanisms in the lateral part of the amygdala, these results may appear unexpected. However, at least in the place-conditioning paradigm, the repeated withdrawal treatment differed from electrical kindling of the basolateral amygdala, perhaps suggesting that these treatments affect different parts of the system. Repeated withdrawal also differed from electrical kindling of the BLA in its effects on fear conditioning<sup>16</sup>. Thus, the behavioural changes we see following repeated withdrawal may not involve basolateral amygdala. There is evidence that the central amygdala is also involved, independently of the lateral aspects, in fear conditioning<sup>26</sup>, and it seems possible, given the pattern of deficits seen in appetitive conditioning<sup>25</sup>, that repeated withdrawal-induced impairments in fear conditioning results reflect changes in plasticity in that area.

### **Repeated Withdrawal from Benzodiazepines**

Since benzodiazepines possess some pharmacological properties in common with alcohol (facilitation of transmission via GABA<sub>A</sub> receptors), themselves possess dependence liability, and give rise to similar subjective effects in human social drinkers<sup>27</sup>, we have also investigated the consequences of repeated withdrawal from chronic benzodiazepine treatment. We have reported that, in mice chronically treated with diazepam, using a method achieving high, and consistent levels of receptor occupancy<sup>28,29</sup> repeated experience of spontaneous withdrawal increased seizure sensitivity and increased the severity of flumazenil-precipitated withdrawal-induced anxiety<sup>30</sup>, indicating similarities with repeated alcohol withdrawal. These are the first demonstrations that repeated bouts of withdrawal from benzodiazepines are potentially deleterious. In keeping with the evidence that repeated experience of withdrawal from ethanol results in impaired aversive conditioning, we found that previous experience of benzodiazepine withdrawal weakens the ability of a withdrawal experience to act as an unconditioned aversive stimulus in a conditioned taste aversion paradigm<sup>31</sup>, and prevents the withdrawal experience from inducing *c-fos* expression in accumbens shell<sup>31</sup>. We tested the possibility that the failure of the withdrawal to support taste aversion conditioning might be accounted for by a phenomenon described in learning theory, the US pre-exposure effect, but found no evidence that this was the case<sup>32</sup>. A further experiment<sup>33</sup> suggested that repeated withdrawal might induce a form of "learned helplessness", an inability to learn about aversive events. Re-evaluation of the data from this experiment indicates that repeated withdrawal experience following the initial conditioning procedure did not block the expression of an already acquired conditioned taste aversion, consistent with the lack of effect of repeated alcohol withdrawal on expression of conditioned fear. Thus the data from repeated diazepam withdrawal are compatible with repeated withdrawal from sedative hypnotics more generally inducing deficits in acquisition of associative conditioning. (It would also suggest that using BZs during ethanol withdrawal to block convulsions will not prevent the longer term sequelae of repeated withdrawal). These results imply that repeated attempts at withdrawing from benzodiazepine treatment may be harmful, but we are unaware of clinical investigations which might substantiate this possibility. It would be of interest to discover whether those patients who are severely dependent upon benzodiazepines may have more previous attempts at withdrawal than those who are less severely dependent despite a similar history of drug taking.

### **Mechanisms of Adaptation to Alcohol and Benzodiazepines, and Implications for Plasticity**

We have made less satisfactory progress in understanding the mechanisms whereby repeated alcohol or benzodiazepine withdrawal achieve their longer term effects in predisposing to seizure activity, and impairing conditioning. Our finding that long term potentiation in both hippocampal CA1, and lateral amygdala pathways is impaired by a single withdrawal, and this effect is exacerbated following three withdrawals<sup>8</sup>, points to a potential mechanism whereby learning may be impaired. If the reduction in capacity for LTP comes about through prior saturation of synapses, then this observation could also explain heightened seizure sensitivity following repeated withdrawal.

#### ***Glutamate Receptor-mediated Plasticity***

The mechanisms underlying long term potentiation have been studied extensively in the literature, and several different kinds of plasticity exist. In principle, any of these might form the basis of withdrawal-induced plasticity. Probably the best-known form of plasticity depends on activation of glutamatergic NMDA receptors, the resulting calcium influx inducing both short term, and long-term changes in expression of AMPA receptors, which facilitate subsequent transmission through enriched synapses. In collaboration with [REDACTED] we have obtained evidence that a single episode of withdrawal from chronic benzodiazepine treatment causes an increase in [3H]-Ro 48 8587 (AMPA ligand) binding sites in several brain regions, including amygdala<sup>34,35</sup>; such increases in AMPA receptor binding were not found following repeated withdrawal,

providing a neurobiological correlate of reduced aversiveness of withdrawal following previous withdrawal experience<sup>34,35</sup>). We have also extended these observations using *in situ* hybridization methods to show decreased expression of message for GluR1 (but not GluR2) subunits of AMPA receptors in cortical regions following a single or repeated withdrawal, though in the basolateral amygdala decreases were seen only following repeated withdrawal. It is not clear to us why increased binding of an AMPA receptor ligand is accompanied by reduced expression of GluR1 subunit mRNA, since there is no evidence that the radiolabel possesses differential affinities for receptors containing different subunits. However, a potential importance for GluR1 subunit regulation by benzodiazepine withdrawal is suggested by the observation that GluR1 knockout mice do not show a conditioned taste aversion when a novel taste is paired with flumazenil-induced withdrawal from benzodiazepine (in preparation). Thus downregulation of GluR1, either by targeted gene deletion, or by repeated withdrawal from a benzodiazepine, is associated with impaired aversive conditioning.

An obvious test of the involvement of NMDA receptors in withdrawal-induced plasticity is to block the receptor pharmacologically during withdrawal, and subsequently to test behaviours normally susceptible to the withdrawal-induced plasticity. We found that administration of the competitive NMDA antagonist, CGP39551, to mice during periods of repeated withdrawal did not protect them against subsequent PTZ-induced seizures, nor anxious like behaviour during withdrawal<sup>36</sup>. Indeed, such treatment increased the severity of subsequent withdrawal seizures. Nor was CGP39551 effective as an anticonvulsant when given immediately before withdrawal-seizure induction. These observations suggest that NMDA receptor-mediated mechanisms are not important in the alcohol withdrawal-induced plasticity which contributes to increased anxiety and sensitivity to seizures (though alcohol seems to differ from benzodiazepines in this respect<sup>37</sup>).

Alternative means of mediating plasticity include increased calcium flux through voltage-gated L-type calcium channels, or through  $\text{Ca}^{2+}$ -permeable AMPA receptors (receptors not containing the GluR2 subunit), or through enhanced release of glutamate. Available AMPA antagonists are unsuitable for these kinds of experiment because of their short half-life and relatively poor brain penetration. Since we have colonies of mice in which either the GluR1 or the GluR2 subunit of AMPA receptors has been deleted, we intend to use these to investigate potential roles of AMPA receptors in repeated withdrawal sensitisation. We have shown in a parallel, BBSRC-funded project, that deletion of the GluR1 subunit gives rise to a behavioural syndrome closely similar to that seen in rats with lesions of the basolateral amygdala<sup>38,39</sup>, while GluR2 knockouts display a pattern of deficits in appetitive conditioning more closely resembling rodents with central amygdala nucleus lesions<sup>40</sup>. Thus, according to our working hypothesis, behavioural changes following repeated withdrawal from alcohol may be more likely to be affected by GluR2 deletion than GluR1. In initial experiments we have found that the GluR1 knockout mice did not differ from wildtype controls in the extent to which withdrawal from alcohol presensitised them to PTZ-induced seizures, which is consistent with the hypothesis, and we will begin to investigate the GluR2 knockouts within the next few months. From our previous work, it seems possible that deletion of GluR2 subunits may exacerbate the effects of repeated withdrawal, since this manipulation, like repeated withdrawal, impairs Pavlovian-to-instrumental transfer<sup>40</sup>, and impairs acquisition of conditioned fear<sup>41</sup>.

### *Nitric Oxide*

A more recently described form of synaptic plasticity involves the gaseous transmitter nitric oxide (NO). We have begun to study the acute effects of alcohol on NO, and found that expression of the neuronal NO synthase (NOS) isoform is significantly up-regulated in response to alcohol intake<sup>42</sup>. At the cellular level, the numbers of NADPH-d positive cells was increased transiently in the nucleus accumbens and amygdala, 8 hours after a single dose of ethanol, returning to control levels 24 hours later. The transient up-regulation is also observed for nNOS protein levels, which increased significantly 8 hours, returning to basal levels at 24 hours, post withdrawal. At the level of gene expression, a significant increase in nNOS gene expression occurs 8 hours after alcohol and the basal level is restored 24 hours after injection<sup>42</sup>. Following 21 days treatment with alcohol in the diet, similar effects were seen 8h into withdrawal. Alcohol intake results in marked, systematic and coherent up-regulation of NO signaling. This suggests that the NO signaling pathway may have a role to play in the brain's adaptive response to alcohol.

### *Tissue Plasminogen Activator*

Synaptic remodelling associated with LTP and with electrical kindling, is associated with a cascade of events initiated by calcium influx, and which include induction of a number of immediate early genes (i.e.g.). One such i.e.g. encodes the serine protease, tissue plasminogen activator (tPA), which is secreted into the extracellular space where it is thought to play a role either in directly remodelling synaptic connections, or indirectly by

converting plasminogen located in microglia, into a second serine protease, plasmin, which is responsible for synaptic remodelling.

Mice that lack the gene for tPA (tPA knockout mice, tPA<sup>-/-</sup>) are impaired in acquiring a DRL (differential reinforcement at low rates of responding) task, a task which depend upon upon intact functioning of the hippocampus and prefrontal cortex<sup>43</sup>; this deficit is reversed by signaling the availability of the reinforcer<sup>44</sup>. We suggest that interactions of tPA with NMDA receptors contribute to such deficits<sup>45</sup>. Repeated ethanol withdrawal completely abolished the ability of the knockout (but not the wildtype) to acquire a DRL task, an effect not achieved by a single withdrawal (in preparation).

tPA<sup>-/-</sup> mice were more sensitive to ethanol, both at acute stimulant and sedative doses. However, these mice were less sensitive to the rewarding properties of ethanol, seen as a decrease in ethanol self-administration and an inability of low doses of ethanol to induce conditioned place preference in tPA<sup>-/-</sup> mice. Ethanol is known to induce tPA in the periphery, so that increased tPA expression might represent a compensatory mechanisms in response to intoxication. However, we have not yet found evidence for ethanol-induced induction of tPA in the brain (at least in cerebellum; unpublished).

## Conclusions

Our findings under the grant have considerably deepened knowledge of the consequences of repeated withdrawal from ethanol, and opened up a new area of research. We have developed a novel hypothesis to account for the consequences of repeated withdrawal in increasing seizure sensitivity and responses to anxious stimuli, accompanied by impaired learning about aversive events, and have provided a first set of electrophysiological, behavioural and immunohistochemical evidence in support. While we have made a number of interesting observations regarding neurobiological events accompanying repeated withdrawal, these observations are not yet integrated into a coherent account.

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