Behavioural and Neurobiological Effects of Repeated Ethanol Withdrawal

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I hereby declare that this thesis has not been and will not be submitted in whole or in part to another University for the reward of any other degree.

Signature ……

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Investigations into the behavioural and neurobiological effects of repeated ethanol withdrawal

Summary

This thesis presents a rat model, by which the effects of repeated ethanol withdrawal on withdrawal severity was investigated, in relation to the cognitive and behavioural deficits associated with repeated episodes of withdrawal. Repeated ethanol withdrawal in the rat has been well established to model the effects of repeated episodes of human alcohol detoxification. This model has enabled the study of withdrawal severity and the role of the prefrontal cortex in the form of rat behaviour.

Chronic ethanol consumption led to disrupted circadian rhythm especially in measures of wakefulness and NREM sleep. However, there were no cumulative effects of multiple ethanol withdrawals. These results were confounded by altered circadian rhythms observed in the control group.

Repeated ethanol withdrawal induced significantly higher levels of C-Fos, a marker of neuronal activation, compared to a single withdrawal episode. In addition, repeated ethanol withdrawal also induced significantly higher Zif 268 expression, a marker for neuronal plasticity, in the prelimbic cortex. These findings indicated a sensitivity of prefrontal cortical areas in response to repeated ethanol withdrawal.

In assessing performance on a 2-choice serial reaction time task, repeated ethanol withdrawal resulted in more sessions to criterion, indicating possible learning deficits but only when the withdrawal experience occurred prior to behavioural training and testing.

Repeated ethanol withdrawal did not significantly impair attentional set shifting ability on the intradimensional / extradimensional task. The findings of this current thesis suggest that repeated ethanol withdrawal did not produce significantly severe cognitive deficits as measured by behavioural tasks sensitive to prefrontal cortical damage, despite neurobiological activation of prefrontal areas.

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Chapter 1

General Introduction

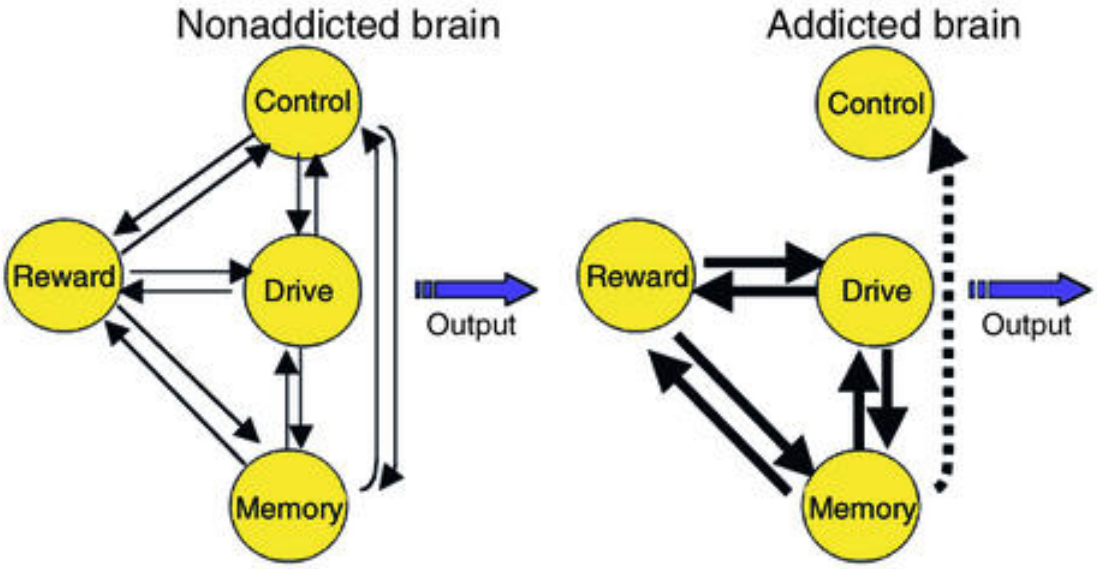
1.1 Alcohol Abuse:

Drug addiction is defined as a chronic relapsing disorder consisting of two major characteristics: a compulsion to take the drug with a "narrowing behavioural repertoire towards excessive consumption" and compulsive drug use characterised by loss of control over drug consumption (DSM 1994). Alcoholism is characterised by excessive consumption of alcohol, the development of tolerance and withdrawal and impairment in social and occupational functioning (DSM 1994).

Addictive behaviour follows a cyclical pattern, whereby an individual's "reward" system, which responds to natural rewards determined by internal motivational states such as hunger, thirst or sexual arousal, is hijacked by the actions of the addictive drug on the brain. Addictive drugs produce patterns of behaviour which dominate an individual's motivation for all other rewards, eventually resulting in compulsive drug taking which is the hallmark of drug addiction. Like natural rewards, addictive drugs are sought in anticipation of positive outcomes, but with continued and repetitive use, the brain produces homeostatic adaptations that give rise to drug dependence which can result in distressing, severe and sometimes life threatening withdrawal symptoms on cessation of drug use. Although it has been theorised that drug addicts continue drug taking as a method of avoiding withdrawal symptoms (Koob and Le Moal 1997; Hutcheson, Everitt et al. 2001), this motivation alone does not explain addiction (O'Brien, Childress et al. 1998; Berke and Hyman 2000). In animal studies, reinstatement of drug self-administration after drug cessation is more potently motivated by drug re-exposure than by withdrawal (O'Brien, Childress et al. 1998). It would indicate that the escalating stages of drug dependence and subsequent drug withdrawal cannot explain the persistence of relapse risk which can still exist long after

detoxification (Wikler and Pescor 1967; O'Brien, Childress et al. 1998; Berke and Hyman 2000).

It is important to stress that drug addictions, including alcoholism, are not static in nature but consist of different components that comprise a cycle. The addiction cycle, as seen in figure **1.1** is initiated with drug intake or experimentation, which represents the first stage of an individual's failure to self-regulate behaviour. Repeated drug use leads to significant brain changes which influence brain circuits involved in motivation, reward, drive, salience attribution, inhibitory control and memory consolidation (Volkow, Fowler et al. 2003). Hence by perpetuating this cycle of addictive behaviour, repeated failure to self regulate drug taking behaviour leads to additional negative affect (Baumeister 1994). In Volkow's addiction model of *impaired response inhibition and salience attribution* (I-RISA syndrome of drug addiction), it is proposed that impaired response inhibition plays a crucial role at every stage of the drug addiction cycle including drug intoxication, drug craving, bingeing and drug withdrawal.



Adapted from Volkow, Fowler and Wang (2003)

Figure 1.1. depicts a model proposed by Volkow and associates, which details the four circuits involved in drug addiction, namely reward, motivation / drive, memory and control circuits. All these circuits interact with each other. During addiction, there is more reward value attributed to the drug, which activates the reward, motivation and memory circuits which work together to surmount the inhibitory control exerted by the control circuit located in the prefrontal cortex. This creates a positive feedback loop, initiated by drug intake and maintained by the enhanced activation of the motivation and memory circuits. Diagram adapted from Volkow, Fowler and Wang (Volkow, Fowler et al. 2003).

Addiction scientists have established that although chronic drug exposure might be a pre-requisite for the development of addiction, its manifestation is a function of interacting drug effects, biological and environmental factors. The harm caused by sustained consumption of ethanol has been reported to be second only to heroin consumption (Nutt and Peters 1994; Nutt, Lingford-Hughes et al. 2003). Alcohol abuse and addiction is a health issue as well as a social issue, affects the health of the individual and presents a host of societal problems. Alcohol abuse can lead to numerous medical conditions such as cirrhosis of the liver, heart disease, pancreatitis, Korsakoff's dementia and foetal alcohol syndrome.

Hence, by piecing together the different components of alcohol addiction, we can hopefully glean a better understanding of this brain disease. The work of this thesis hopes to contribute to the existing literature by investigating the role of alcohol withdrawal on behaviours mediated by the prefrontal cortex.

1.2 A History of Alcohol Use:

Ethanol (or ethyl alcohol) is produced by fermentation, a natural process which occurs when yeast cells come into contact with sugar, usually from fruit or grain. The yeast converts each sugar molecule into two molecules of alcohol and two molecules of carbon dioxide. The fermentation continues until the alcohol concentration reaches approximately 15%, at which point, the yeast is killed by the high alcohol content. The natural process of alcohol production more than likely explains the reason that alcohol consumption is as age old as civilisation itself.

Cultures all over the world have discovered alcohol and made it their own, be it wine, believed to have originated as early as c.6000 B.C., beer which was first made popular in the Mesopotamian civilisation in c. 3000-2000 B.C., or spirits, the distillation of which was first described in Salerno, Italy, in 1100 AD. During the middle Ages, alcohol was the beverage of choice when water supplies were commonly contaminated, which earned it the title “aqua vitae” or water of life.

Alcohol has a long history and its use has become intertwined with everyday life as a “social lubricant, sophisticated dining companion, cardiovascular health benefactor or agent of destruction.” (Vallee 1998). Evidence of excessive alcohol consumption can be traced back to ancient Greece and attitudes to excessive drinking can be found in Plato’s Proposed Law on Alcohol Consumption:

“Shall we not pass a law that, in the first place, no children under eighteen may touch wine at all, teaching that it is wrong to pour fire upon fire either in body or in soul…and thus guarding against the excitable disposition of the young? And next, we shall rule that the young man under thirty may take wine in moderation, but that he must entirely abstain from intoxication and heavy drinking. But when a man has reached the age of forty, he may join in the convivial gatherings and invoke Dionysus, above all other gods, inviting his presence at the rite (which is also the recreation) of the elders,

which he bestowed on mankind as a medicine potent against the crabbedness of Old Age, that thereby we men may renew our youth, and that, through forgetfulness of care, the temper of our souls may lose its hardness and become softer and more ductile." (Plato 666b)

Alcohol consumption has become imbued with connotations of healing and has, in the course of history, been widely marketed as "tonics" or "elixirs". This preoccupation with alcohol and the intoxication that arises as a consequence of consumption was further fuelled by the invention and later on, mass distillation of gin or *Jenever* by the Dutch from the mid-1500's (Dillon 2004; Bober 2010). What arose was the Gin craze during the mid 18th Century, when gin was introduced into England during a time of social upheaval and alcohol abuse was a cheap means of coping with poor living conditions amongst the working poor classes. These scenes of drunkenness, violence, madness, starvation and infanticide were characterised by William Hogarth's famous engraving *Gin Lane* in 1751, which served to warn people of the dangers of cheap alcohol and the easy slide down the scale of moral society. In today's society, where binge drinking is highly prevalent amongst, but not restricted to, the young population (16-25 years), the dangers of excessive alcohol consumption are still as relevant as they were back in Hogarth's time, during the reign of George II.

1.3 Epidemiology of Alcohol Abuse:

Alcohol misuse has been defined by the Department of Health as alcohol consumption which exceeds the guideline limits set by the Department, and is currently recommended that men do not exceed 3-4 units and women do not exceed 2-3 units of alcohol per day (Department of Health 1995). This kind of alcohol consumption can progress to increasingly deleterious forms of alcohol misuse, which involve evidence of alcohol-related problems, displaying an increased tolerance of alcohol, withdrawal symptoms and loss of control of drinking (Department of Health 2008). According to a report by the Office of National Statistics on alcohol-related deaths in the United Kingdom between 1991 – 2008, alcohol-related deaths have steadily increased, involving significantly more males than females. In 2008, there were 18.7 deaths per 100,000 in men and 8.7 deaths per 100,000 in women (ONS 2010). In a report by the National Audit Office for the Department of Health on reducing alcohol harm in 2008

(ONS 2010) more than 10 million people (31 % men and 20 % women) regularly exceed the alcohol consumption guidelines set by the Government. Hospital admissions for 3 of the primary alcohol-related conditions (alcohol-related liver disease, mental health disorders linked to alcohol and acute intoxication) have more than doubled in the 11 years between 1995-6 and 2006-7 from 93,459 to 207,788. There were 8,758 deaths from alcohol-related causes in the UK in 2006 which has doubled since 1991. The Department of Health has estimated that alcohol misuse costs the health service £2.7 billion per year. Hence, not only is the cost of alcohol misuse expensive to the National Health Service, but wider costs for society such as crime and disorder, social and family breakdown and sickness absence are also consequences of alcohol misuse.

Of the people who have tried alcohol at least once, 15 % become alcohol-dependent (Anthony et al 1994). Gender differences appear to have an effect on the likelihood of developing an addiction to alcohol as men are more likely than women to become addicted to alcohol (Anthony and Echeagaray-Wagner 2000; Warner, Canino et al. 2001). A study by Anthony et al (Anthony 1994) reported that the estimated peak age for becoming dependent upon alcohol were found to be between ages 17-18 years, in comparison to the peak age for developing cocaine dependence which was at ages 23-25 years. Although cocaine dependence demonstrates a more explosive dependence profile, 12-13% of alcohol users develop alcohol dependence within the first 10 years of alcohol use and the risk of developing alcohol addiction continues through the middle years of adult life (Eaton, Kramer et al. 1989; Wagner 2002). The risk of developing alcohol dependence is becoming increasingly prevalent as binge consumption of alcohol becomes more acceptable amongst young social drinkers. A recent national cross-sectional survey indicate that 46% of men and 30% of women aged 25-44 years exceed the Government-recommended levels of ethanol consumption, figures rising to 49% in men and 39% in women amongst 16-24-year-olds (Walker 2001) revealing that males are more likely to become binge drinkers than females throughout adulthood. Although binge drinking patterns of behaviour change as individuals grow older, teenage alcohol consumption is correlated with a higher likelihood of binge drinking in adulthood (Jefferis, Power et al. 2005). Those who binge drink during their teens and early 20’s are more likely to continue this pattern of

alcohol misuse into their 40's (Jefferis, Power et al. 2005). Alcohol use in binge consumption patterns represents a public health concern (Dyer 2004; Pearson 2004) and there is increasing government focus on policies to help reduce binge alcohol consumption in young binge drinkers (The Cabinet Office 2004).

In 1981, Cloninger and colleagues proposed a hypothesis concerning type 1 and type 2 alcoholism, in order to address the possible genetic basis for alcoholism (Cloninger, Bohman et al. 1981). Type 1 alcoholism is thought to be relatively late in onset (after 25 years of age) and affects both males and females. The personality traits of type 1 alcoholics include high harm avoidance; in other words, type 1 alcoholics are cautious, inhibited, more likely to worry and experience feelings of guilt over their alcoholism (Cloninger 1987). They are also less likely manifest the personality trait of novelty seeking and use alcohol for anti-anxiety purposes and are more prone to binge patterns of alcohol consumption (Vaillant 1994).

Type 2 alcoholism, by Cloninger's definition was thought to affect primarily males and to manifest itself before the age of 25 (early onset alcoholism) and is characterised by violence and illegal activities both with or without alcohol use. The personality traits of type 2 alcoholics demonstrate little need for social approval, lack of inhibition and high novelty seeking (Bohman, Cloninger et al. 1987). Such individuals tend to use alcohol for its euphoric effects and this type of alcoholism is hereditary in males. Cloninger at al's hypothesis on the two types of alcoholism arose from clinical observations that alcoholism in individuals with alcoholic relatives have an earlier age onset, have a worse recovery prognosis and is more severe (Tarter, McBride et al. 1977; Goodwin 1979; Cloninger, Bohman et al. 1981; Penick, Powell et al. 1987). The two types of alcoholics indicate that some individuals demonstrate a predisposition towards a certain pattern of alcohol abuse which results in different types of behaviours. This differentiation of type 1 and type 2 alcoholics highlights the contribution of genetics to the development of alcoholism.

1.4 Pharmacology of alcohol:

Alcohol is rapidly and completely absorbed into the bloodstream from the stomach and gastro-intestinal tract. Metabolism of alcohol occurs predominantly within the

liver by means of alcohol dehydrogenases (Nutt and Peters 1994) before its products are distributed throughout the bodily fluids. Some studies suggest that women are more susceptible to the effects of ethanol than men, (Baraona et al 2001, Ceylan-Isik et al 2010), possibly due to less gastric metabolism of ethanol in women than in men (Baraona et al 2001, Frezza et al 1990).Women have also been found to suffer more severe brain and organ damage following binge or chronic ethanol consumption (reviewed by Ceylan-Isik et al 2010). Alcohol has a wide variety of actions of neuronal transmission. It works primarily on the central nervous system as a depressant. The alcohol molecule exerts its actions on four main sites of importance: the movement of sodium (Na^+) ions across cell membranes, the binding of gamma-aminobutyric acid (GABA) to its receptors, the responsiveness of NMDA receptors and activity at opiate receptors (Klein 2007). Alcohol inhibits the movement of Na^+ ions across the cell membrane, an effect which is dose dependent, i.e. the higher the alcohol dose, the greater the reduction of CNS function. Decreased CNS function leads to diminished judgement, and impaired motor coordination, symptoms typical of alcohol intoxication. Numerous textbook references state that alcohol also acts on the $GABA_A$ receptor, the action of which mediates the flow of chloride (Cl^-) ions into the cell and leads to the generation of inhibitory postsynaptic potentials (IPSPs) by binding to the $GABA_A$ receptor, although alcohol's direct actions on the $GABA_A$ receptor remain controversial. Olsen and colleagues have proposed that alcohol, even at low doses, acts directly at the $GABA_A$ receptor, through the δ subunit (Sundstrom-Poromaa, Smith et al. 2002; Wallner, Hanchar et al. 2003; Hanchar, Wallner et al. 2004; Olsen, Hanchar et al. 2007; Santhakumar, Wallner et al. 2007) whereas evidence has also been found showing that ethanol does not have a direct mechanism of action on the $GABA_A$ receptor via the δ subunit (Borghese, Storustovu et al. 2006). The disparity between these studies demonstrates the complexity of the role of $GABA_A$ receptor and alcohol effects.

Alcohol appears to disturb the fine balance between excitatory and inhibitory influences on the brain, which result in disinhibition, ataxia and sedation. With chronic use, individuals develop tolerance to the effects of alcohol, leading to dose escalation and withdrawal symptoms on cessation of alcohol consumption.

Acute alcohol has a wide range of behavioural effects. It acts as a sedative-hypnotic and produces dose-dependent behavioural effects such as sedation and hypnosis (sleep induction). Low levels of blood alcohol (approximately 10 - 50 mg /dL) gives rise to personality changes, increased sociability, talkativeness, increased positive mood, confidence and assertiveness. Marginally higher levels of blood alcohol (80 - 100 mg /dL) lead to more pronounced mood swings, emotional outbursts and disinhibition and blood alcohol levels at 150 – 200 mg / dL result in marked ataxia, major motor impairment, staggering, slurred speech and impairment in reaction time (Koob 2006).

1.5 Alcohol's effects on the Central Nervous System:

All drugs of abuse exert their physiological effects through various mechanisms of action. However one molecular mechanism of action that all drugs of abuse share is that they all increase dopamine levels in terminal areas of the mesolimbic dopamine reward pathway in the brain (Nestler 2005). For instance, amphetamine and cocaine act directly in the dopamine synapse in the terminal areas of the mesolimbic dopamine pathway, whereas alcohol, barbiturate and benzodiazepines act by disinhibiting the mesolimbic dopamine pathway by their actions on the $GABA_A$ receptor (Wise 1980). This brain pathway extends from the ventral tegmental area to the nucleus accumbens, which plays a central role in the reinforcing effects of addictive drugs including alcohol. Although cocaine's actions on the mesolimbic dopamine pathway from the VTA to the nucleus accumbens occurs on primarily dopaminergic neurones, Dixon and colleagues propose that the nucleus accumbens, which contain primarily GABAergic neurones, feeds back to the VTA, by activation of medium spiny neurones (Dixon, Morris et al. 2010). Thus, intra-accumbal GABAergic systems are thought to regulate incentive motivational outputs, which lead to compulsive behaviours including drug abuse. However the mesolimbic pathway does not operate in isolation; it is closely associated to the mesocortical pathway which also originates in the VTA and sends dopaminergic projections to the prefrontal cortex (Koob 2006). It is through these actions in both mesolimbic and mesocortical circuits that chronic ethanol consumption exerts actions to produce cognitive and behavioural impairments.

1.6 Repeated ethanol withdrawal in humans:

Alcoholism is defined as a chronic relapsing disorder, characterised by compulsive patterns of alcohol consumption. It is well established that alcoholics are more susceptible to withdrawal-induced seizures if they have experience of previous detoxifications compared with alcoholics experiencing their first detoxification from alcohol (Gross, Rosenblatt et al. 1972; Ballenger and Post 1978; Brown, Anton et al. 1988; Lechtenberg and Worner 1991; Lechtenberg and Worner 1992; Booth and Blow 1993). It has been hypothesised that the correlation between repeated episodes of ethanol withdrawal and increased seizure susceptibility occurs as a consequence of adaptation of brain mechanisms in response to multiple withdrawals, similar to that occurring during the epileptic kindling process.

Kindling is an electrophysiological phenomenon which occurs in the brain and was initially proposed by Goddard et al (Goddard, McIntyre et al. 1969) who found that repeated bipolar electrical stimulation of loci associated with the rat limbic system (but not from stimulation of many other regions of the brain) led to permanent changes in brain function and by decreasing seizure thresholds led to increased chance of eliciting convulsions. Although the kindling effect has not been confirmed in human alcoholic brains, this hypothesis does appear to be a plausible explanation for the progressive worsening withdrawal symptoms experienced by alcoholics undergoing repeated episodes of alcohol detoxification.

Several clinical studies have demonstrated withdrawal effects in human alcoholics which could be explained by the kindling hypothesis. For instance, in a study of 200 men who had experience of ethanol withdrawal, Ballenger and Post (Ballenger and Post 1978) found an association between more serious withdrawal symptoms and a longer duration of alcohol abuse, which was independent of age. From this study, Ballenger and Post proposed limbic kindling as a mechanism for the progressive worsening of withdrawal symptoms. In a retrospective chart review study, Brown at al (Brown, Anton et al. 1988) found that 48% of alcoholics who experienced withdrawal seizures had experienced at least 5 previous detoxifications compared to only 12% of a control group (i.e. alcoholics who did not experience withdrawal seizures) and concluded that the number of previous withdrawals and the early age of the first

detoxification are critical factors that predispose individuals to withdrawal-induced seizures.

Using an observation study of 400-500 patients, Lechtenberg and Worner (Lechtenberg and Worner 1990; Lechtenberg and Worner 1992) found a correlation between seizure prevalence and multiple detoxification hospital admissions. These studies, taken together, reveal a strong correlation between increasing severity of withdrawal symptoms and multiple detoxifications.

It can be postulated that this increasing severity of withdrawal symptoms can be extended to the cognitive abilities which may reveal impairments as a consequence of multiple detoxifications. The amygdala, a limbic brain structure which is involved in emotion and fear conditioning shows functional impairment in relation to repeated detoxifications. Alcoholics who have undergone more than two medically supervised detoxifications experienced more interference from words associated with emotional experience from alcohol (Duka, Townshend et al. 2002). This study used an emotional Stroop task in which social drinkers were compared with alcoholic patients, and asked to name the ink colour of positive and negative emotional words that were associated with alcohol effects, whilst attempting to ignore the meaning of the words. Alcoholics with prior experience of two or more withdrawals made more errors when naming the ink colour of emotional words compared to social drinkers. This finding demonstrates that alcoholics are susceptible to greater interference from the semantic meaning of words, which is indicative of a higher emotional sensitivity compared to social drinkers. Alcoholics also detect more fear in all facial expressions, indicating the emotional sensitivity observed in alcoholics in the emotional Stroop task can be observed outside of alcohol-related contexts (Townshend and Duka 2003). These clinical studies suggest that multiple ethanol detoxifications arise from impaired function of the amygdala, which lead to enhanced fear recognition, a finding which is supported by the animal work of Pinel et al (Pinel, Van Oot et al. 1975) who have reported that periodic electrical stimulation of the rat brain can result in the intensification of the ethanol withdrawal syndrome, and subsequent ethanol withdrawals were also greatly increased in severity with repeated electrical stimulation. Stephens and colleagues (Stephens, Brown et al. 2001) have proposed that learning deficits in relation to fear

conditioning observed in multiply withdrawn rats may be due to dysfunction of the amygdala, which is supported by the finding of impaired transmission in the amygdala as a consequence of multiple ethanol withdrawal (McCown and Breese 1990).

Brain damage as a repercussion of alcohol abuse is well documented (Moselhy, Georgiou et al. 2001) and prior alcoholic detoxifications may accelerate disruption of amygdala function (Adinoff 1994; Stephens, Brown et al. 2001). It is thus plausible to suggest that repeated detoxifications may lead to impaired cognitive function through damage to the amygdala and its connections, implicating possible damage to the prefrontal cortex.

As previously discussed, the frontal lobes are particularly sensitive to the deleterious effects of chronic alcohol abuse (Moselhy, Georgiou et al. 2001), evidence for which is provided by animal studies, which reveal increased brain damage after repeated ethanol withdrawal or when repeatedly high amounts of alcohol in the brain are followed by periods of abstinence, as in the case of binge alcohol consumption (Veatch and Gonzalez 1999; Crews, Braun et al. 2001; Penland, Hoplight et al. 2001).

Further evidence for the relationship between cognitive impairments and alcohol detoxification comes from tests of high order cognitive function such as the Porteus maze, used to measure the ability to accomplish goals, the vigilance task which measures the ability to inhibit a prepotent response, and the delay task in which a subject is required to wait before making a response to receive a reward. Duka and colleagues (Duka, Townshend et al. 2003) found alcoholic patients took more time to complete and made more errors in the maze tasks and more commission errors in the vigilance task. Patients with two or more detoxifications were more impaired in the maze task, the vigilance task and the delay task than patients with a single detoxification or no previous detoxifications which shows alcohol withdrawal leads to impaired cognitive functions which become progressively worse with multiple detoxifications, although factors such as age of onset of heavy drinking and years of problem drinking contribute to the cognitive impairments observed in alcoholic patients.

1.7 Repeated ethanol withdrawal in rats:

Much of the current knowledge regarding the mechanisms of addiction has been derived from studying animal models of addiction. Although no single animal model has been created to emulate the disorder of addiction as a whole, animal models do allow the study of different stages of the addiction cycle, different symptoms of addiction, different psychological constructs involved in addiction, i.e. positive and negative reinforcement. Drug addiction progresses from impulsivity to compulsivity in a cycle which comprises 3 main stages, namely (1) preoccupation with / anticipation of the drug, (2) binge / intoxication and (3) withdrawal / negative affect (Koob 2008). In this thesis, the focus is firmly placed on alcohol withdrawal. Alcohol withdrawal has been well characterised by a states of hyperexcitability in the central nervous system, which results from a previously depressed central nervous system from chronic alcohol use. Alcohol withdrawal in the human can occur up to 36 hours after cessation of alcohol intake (early stages of alcohol withdrawal), which can result in tremor, insomnia, anxiety, anorexia, elevated sympathetic responses including increased heart rate, blood pressure and body temperature. Late stages of withdrawal if untreated, can lead to severe tremor, delirium tremens, vivid hallucinations, high fever and seizures (Koob 2008). As previously discussed, if an individual experiences withdrawal repeatedly, this may not only lead to life-threatening seizures but also to functional impairment of cognitive abilities.

Our well-established rat model of repeated ethanol withdrawal involves feeding male Lister hooded rats a nutritionally complete liquid diet as their sole food source. The three treatment groups receive either control liquid diet or liquid diet containing 7% ethanol. The rats receiving ethanol-containing liquid diet are fed for either 24 consecutive days (single ethanol withdrawal- SWD) or for 30 days with 2 intermediate periods of ethanol withdrawal lasting 3 days, starting at day 11 and day 21, during which the rats are fed control liquid diet (repeated ethanol withdrawal- RWD). All rats are fed rat chow at the end of their liquid diet treatment period. It is important to note the amount of control diet was restricted to the mean amount of ethanol-containing liquid diet that the animals had consumed on the previous day. One possible criticism of this method of alcohol administration is that alcohol levels attained from liquid diet

consumption can occasionally be low. Rats generally find alcohol aversive and will avoid it. As the ethanol is administered via a liquid diet, if the rats are sufficiently hungry, they will consume ethanol but there is no external control over how much ethanol rats consume, in the manner that intraperitoneal injections can achieve stable blood ethanol concentrations.

This model of repeated ethanol withdrawal has been found to produce many of the behaviours observed in human alcoholics who have prior detoxification experience. In particular, rats that have experienced multiple withdrawals show impaired fear conditioning (Stephens, Brown et al. 2001) which possibly demonstrates disrupted functioning of the amygdala (McCown and Breese 1990; Adinoff 1994; Stephens, Brown et al. 2001). Furthermore, animal studies have found that multiple withdrawals impair the acquisition of conditioned fear but had no effect on the expression of conditioned fear (Ripley, Brown et al. 2003). This operant-based study by Ripley et al (2003) involved the presentation of a cue, associated with a footshock. Control animals learned to suppress responding for food reinforcement in order to avoid footshock, whereas ethanol treatment and withdrawal blocked suppression of responding and extinction of conditioned fear was also impaired as a consequence of repeated ethanol withdrawal. Extinction is a form of inhibitory learning which involves the suppression of a previously conditioned response. Recent research has implicated a critical role for the prefrontal cortex in the extinction of both fear expression (Powell, Skaggs et al. 2001; Vidal-Gonzalez, Vidal-Gonzalez et al. 2006; Corcoran and Quirk 2007), fear extinction (Herry and Garcia 2002; Milad and Quirk 2002; Gonzalez-Lima and Bruchey 2004), and drug seeking behaviours. In particular, it appears that the prefrontal area implicated in fear and addiction circuits is the medial prefrontal cortex (mPFC) (Morgan, Romanski et al. 1993; Morgan and LeDoux 1995; Sotres-Bayon, Cain et al. 2006; Peters, Kalivas et al. 2009). The prelimbic cortex, which forms the dorsal part of the medial prefrontal cortex drives the expression of fear and drug seeking, particularly with regards to relapse (McFarland and Kalivas 2001; Capriles, Rodaros et al. 2003; McLaughlin and See 2003; McFarland, Davidge et al. 2004) whereas the ventral region of the medial prefrontal cortex, namely the infralimbic cortex, suppresses fear expression (Herry and Garcia 2002; Milad and Quirk 2002; Gonzalez-

Lima and Bruchey 2004) and drug seeking (Ciccocioppo, Sanna et al. 2001; McFarland and Kalivas 2001; McFarland, Davidge et al. 2004) after extinction. Judging by the literature concerning the convergence of brain circuits of both extinction of conditioned behaviour and fear expression in the medial prefrontal cortex, taken together with the previous findings in this laboratory in animals (Stephens, Brown et al. 2001; Ripley, Brown et al. 2003) and in humans (Townshend and Duka 2003), these brain circuits in the medial prefrontal cortex may play a role in the behavioural deficits that occur as a consequence of repeated ethanol withdrawal.

Using electrophysiological studies, long-term potentiation is reduced in multiply withdrawn rats. Long term potentiation describes a "long lasting enhancement of synaptic transmission occurring at various CNS synapses following a short (conditioning) burst of presynaptic stimulation, typically at about 100 hertz for 1 second." (Rang 2002). This finding indicates that repeated ethanol withdrawal reduces synaptic plasticity which consequently reduces the capacity for future learning (Stephens, Ripley et al. 2005). Reduced synaptic plasticity, measured using zif268 as a biomarker, is further supported by the finding that the increase in zif268 seen as a result of a single withdrawal experience, was not found after repeated ethanol withdrawal, suggesting that following repeated withdrawal, there is reduced plasticity in the brain (Borlikova, Le Merrer et al. 2006). Borlikova et al (2006) suggested a possible reason for this finding was that a single withdrawal non-specifically strengthens weak synapses but after multiple ethanol withdrawals, the pool of synapses available for strengthening with future learning decreases and hence no further plasticity can be used, leading to reduced capacity for long-term potentiation.

1.8 Involvement of the Prefrontal Cortex in Alcoholism:

Scientific research in the field of drug addiction has primarily focused on the biological aspects of addiction. As a consequence, there is an extensive array of information regarding the biological mechanisms involved in the development and maintenance of drug addiction, mainly involving drug-receptor interactions, the effects of chronic drug use, the brain areas involved in addiction and the importance of contextual cues.

There has been considerably less research focused on the cognitive aspect of drug addiction, particularly concerning the prefrontal cortex. The frontal lobes comprise the largest cortical region of the brain, of which the prefrontal cortex (PFC), which constitutes 29% of the total cortex, is the most complex and highly developed region within the human brain (Moselhy, Georgiou et al. 2001). The PFC carries out its function as a massive association cortex, relying on extensive afferent and efferent connections to all other neocortical regions as well as efferent connections to limbic and basal ganglia structures. Volkow and colleagues (2003) proposed that the function of 4 brain circuits are disrupted in drug addiction, namely the reward circuit in the nucleus accumbens and the ventral pallidum, the motivation circuit located in the orbitofrontal cortex, the memory and learning circuit contained within the amygdala and the hippocampus and the control circuit localised to the prefrontal cortex and the anterior cingulate gyrus (Volkow, Fowler et al. 2003). One of the most robust findings from brain imaging studies is abnormalities in the control circuit encompassing the PFC. Disruption of the control circuit is likely to result in impairment of inhibitory control, and decision making is postulated to lead drug addicts to choose immediate over delayed rewards and could contribute to the loss of control of drug intake so characteristic of drug addiction (Goldstein and Volkow 2002). These alterations in brain function are persistent and last long after the cessation of drug use / abuse which renders an abstinent drug addict susceptible to relapse into further drug use.

Long term drug or alcohol exposure can give rise to cognitive dysfunction due to changes in the PFC. The resulting cognitive dysfunction renders an addicted individual unable to inhibit conditioned or unconditioned responses elicited by drugs (Jentsch and Taylor 1999). PFC injury in humans leads to dysfunction in categorisation in cognitive tasks (Andreasen, Nasrallah et al. 1986). Furthermore, PFC damage or dysfunction can result in decreased will and energy, a tendency to engage in repetitive or perseverative behaviour, difficulty in shifting response set and abnormalities of affect and emotion (Hebb 1945; Nauta 1964; Nauta 1971; Drewe 1975; Damasio 1979), as well as deficits in short term memory, planning, problem solving, impulsivity, disinhibition and poor motivation (Kraus and Maki 1997), all of which indicate deficits in executive functions (also known as cognitive control processes). These varied

symptoms demonstrate the high complexity of the frontal lobes and its crucial role in creative thinking, planning of future actions, decision-making, artistic expression, aspects of emotional behaviour, spatial working memory, language and motor control (Miotto, Bullock et al. 1996; Semendeferi, Damasio et al. 1997) and sustaining attention over time (Rueckert and Grafman 1996).

The PFC controls executive functions, which are typified as non-routine, attentionally demanding, volition processes that are involved in goal-directed behaviour (Garavan and Hester 2007). Executive functions commonly investigated in the laboratory include inhibitory control, attention switching, performance monitoring and decision-making. Jentsch and Taylor (Jentsch and Taylor 1999) hypothesised that drug seeking behaviour occurs by 2 related phenomenon, specifically (1) increased incentive motivational qualities of the drug and drug related cues as a consequence of amygdala dysfunction and (2) impaired inhibitory control as a result of frontal cortical dysfunction. Jentsch and Taylor propose that repeated drug consumption may progressively increase impulsivity levels, leading to a greater susceptibility to subsequent relapse. Altered PFC function as a consequence of chronic drug abuse may lead to impairment of inhibitory control processes to guide behaviour. Frontal cortex lesions can result in significant cognitive impairments including disinhibition (Milner 1982) and a preference for small immediate rewards over larger delayed rewards, also known as impulsive choice (Damasio 1996).

Furthermore, frontal cortical dysfunction or reduced dopamine activity in the frontal cortex can activate subcortical dopamine systems (Louilot, Le Moal et al. 1989; Piazza, Rouge-Pont et al. 1991). The functional relationship between the prefrontal cortex and the nucleus accumbens is demonstrated by the observation that animals more susceptible to the acquisition of intravenous self-administration showed decreased prefrontal dopaminergic activity (Piazza, Rouge-Pont et al. 1991).

1.9 Attentional control:

Executive functions play a crucial role at both the beginning and the end of the addiction life cycle. For instance, impaired cognitive function in the form of impulse control may increase an individual's susceptibility to the first impulsive use of an

addictive drug or to the transition from recreational to addictive use, empirical support for which is provided by behavioural and cognitive measures of impulsivity in 10-12 year olds, which were found to predict drug use at the age of 19 (Tarter 2003). At the other end of the addiction spectrum, executive dysfunction may contribute to the risk of relapse in abstinence. Stroop-like tasks which assess the attentional bias for drug-related stimuli, have been shown to predict relapse (Cox, Hogan et al. 2002; Waters, Shiffman et al. 2003). Stroop –like tasks are used to measure attentional biases in which irrelevant evocative information can interfere with a primary task. The essential feature of the Stroop-like tasks is that irrelevant information will prove distracting, resulting in slowing of responses and a reduction in accuracy. Stroop-like attentional biases have been observed in alcoholics (Cox, Hogan et al. 2002; Duka and Townshend 2004; Lusher, Chandler et al. 2004), smokers (Wertz and Sayette 2001; Waters, Shiffman et al. 2003), heroin users (Franken, Kroon et al. 2000) and cocaine users (Hester 2006) which indicates the attentional biases may occur via a common mechanism of drug abuse rather than being attributed to a specific drug action.

1.10 Inhibitory control:

Inhibitory control describes the ability to suppress interfering stimuli, interpretations and memories (Dagenbach 1994). Neuroimaging studies have linked activity in the prefrontal cortex with inhibitory control (Garavan, Ross et al. 1999; Konishi, Nakajima et al. 1999; Menon, Adleman et al. 2001) however, brain activation for these inhibitory functions extends beyond prefrontal regions to encompass the supplementary motor area, pre-supplementary motor area, occipital and parietal lobes (Liddle, Kiehl et al. 2001; Mostofsky, Schafer et al. 2003). Cocaine users have been shown to have poor inhibitory control (Logan 1984; Fillmore and Rush 2002; Fillmore, Rush et al. 2006) in addition to reduced prefrontal activity during response inhibition (Kaufman, Ross et al. 2003; Hester and Garavan 2004). Hester and colleagues have reported that deficits in inhibitory control in cocaine users were more pronounced with increased working memory loads than in control participants.

Inhibitory control can be studied in clinical or non-clinical populations using the go / no go task and the stop signal reaction time task. In a typical go / no go task, after initiation of a trial, the subject is required to respond when cued to do so by a “go”

signal. However, when a “no go” signal is presented, either concurrently with the “go” signal or immediately preceding it, the subject is required to withhold their prepotent responding. The stop task paradigm is similar to the go / no go task except that the “stop” signal is presented after the presentation of the “go” signal. The closer the stop signal is to the moment at which the subject is required to respond, the more difficult it is for the subject to inhibit their response. Another method of measuring inhibitory control in animals is the 5-choice serial reaction time task, in which subjects are required to suppress responses until a stimulus signals that it is appropriate to respond (Carli, Robbins et al. 1983). Although the 5CSRTT was not solely designed to measure impulsivity, successful performance does require an aspect of behavioural inhibition. During the 5CSRTT, the animal is required to make a nose poke response in one of the five nose poke apertures at the time when a stimulus light located within the aperture is illuminated. After the beginning of a trial and before the stimulus light illumination, there is a 5-second inter-trial interval (ITI) during which an animal must withhold its responding in any of the apertures. A response made during the ITI is a premature response and is punished. Premature responding provides a means of measuring motor impulsivity which translates to impaired impulse control or inhibitory control. Poor inhibitory control can lead to higher levels of impulsivity which is thought to play a role in several key transitional phases of drug abuse (Perry and Carroll 2008), and it has been suggested that during the development of drug addiction, there is a shift from impulsive to compulsive drug seeking and taking behaviour (Belin, Mar et al. 2008). Impulsivity has been hypothesised to play a role at every stage of the addiction cycle, namely acquisition, escalation/dysregulation, during abstinence and relapse (Perry and Carroll 2008).

This evidence suggests the prefrontal cortex plays a significant role in inhibitory control, which is disrupted by drug use. One may postulate that the effect of drug abuse on prefrontal cortical function is progressive, resulting in more profound inhibitory deficits with compulsive drug use. During acquisition, individuals may make the impulsive choice to initiate drug use because they place a greater value on immediate euphoric drug effects over larger future benefits such as personal, social, educational and economic success or well being (Madden, Petry et al. 1997; de Wit

and Richards 2004). Individuals with impaired inhibitory control may also be unable to resist environmental cues, such as peer pressure, that lead them to abuse drugs (de Wit and Richards 2004).

An escalating or dysregulated pattern of drug use is another critical phase of addiction and is thought to represent the switch from "control" to "loss of control" in addiction (Koob and Le Moal 2001; Koob and Kreek 2007) which may represent PFC dysfunction. Drug escalation could reflect increased impulsivity either as a consequence of acute or chronic drug effects

1.11 Impulsivity:

Impulsivity may be considered a subdivision of inhibitory control as the definition of impulsivity as put forward by Evenden is "actions that are poor conceived, prematurely expressed, unduly risky, inappropriate to the situation and that often result in undesirable outcomes" (Evenden 1999). Brain mechanisms for impulse control may provide a means by which rapid conditioned responses are suppressed in order for slower cognitive mechanisms to guide behaviour. However, impulsivity is a multifaceted construct and it is important to recognise that impulsivity described in human studies refer to a variety of behaviours including "sensation seeking, risk-taking boldness, adventuresomeness, boredom susceptibility, unreliability and unorderliness." (Depue and Collins 1999). As these behavioural traits of impulsivity have been determined from personality inventories, Whiteside and Lynam (Whiteside 2001) have mapped these complex personality traits which represent behaviour, for instance, "positive" and "negative urgency" which translates in behavioural terms to rash actions in response to positive or negative mood respectively, "lack of planning" which represents acting without forethought, "lack of perseverance" which represents failure to tolerate boredom or to remain focused in the face of distraction, and sensation seeking which translates as the tendency to seek novel or thrilling stimulation.

Dick et al (2010) have discussed the difficulty in making comparisons between laboratory-based performance measures and personality traits, as the former demonstrate specific cognitive processes under experimental conditions whereas

personality traits are stable, independent of particular tests and likely involve broader cognitive functions (Dick, Smith et al. 2010). It is possible that the difficulty in reconciling the task performance in the laboratory with self-reported personality traits which resulted in numerous varieties of impulsivity to be classified as a complex construct. Reynolds and colleagues (Reynolds, Penfold et al. 2008) have suggested that laboratory tasks measuring impulsivity should be categorised into either “impulsive disinhibition”, (including a stop task and a Go / No Go task) and “impulsive decision making” (including delay discounting and Balloon Assessment of Risk Task), a distinction which can also be categorised as “impulsive action” and “impulsive choice”. Either of these can be measured in animal tasks, in which impulsive action measures the inability to withhold a prepotent response and impulsive choice measures intolerance to delay of reward or perseveration of a non-rewarded response. It is imperative to point out that no laboratory based performance measure gives a complete assessment of all types of impulsivity; however, the ones currently employed to measure impulsive behaviours provide a reasonable picture of “impulsive choice” and “impulsive action”.

Although the prefrontal cortex, which plays a crucial role in response inhibition, is thought to be involved in successful performance in the 5CSRTT, there is also evidence which suggests a role for the striatum, a brain area which shows high connectivity to the prefrontal cortex, in impulsivity. Reversal learning, in which a subject is trained to respond discriminatingly to 2 different stimuli through reward and punishment conditions and then subsequently trained under reversed reward values, is disrupted by damage to both the orbitofrontal cortex and the ventral striatum (Divac, Rosvold et al. 1967; Stern and Passingham 1995) which receives its input from the prefrontal cortex primarily from the orbitofrontal cortex and the medial PFC (Haber, Fudge et al. 2000). The striatum has been linked to movement initiation in monkeys (Lebedev and Nelson 1999) and also suppression of movements during anti-saccades (Raemaekers, Jansma et al. 2002) and primed responses in humans (Aron, Schlaghecken et al. 2003) which implicates the striatum in both the initiation and the inhibition of motor responses. The role of the striatum in motor control is supported by clinical studies of neurological illnesses associated with dysfunctional motor control, e.g. Parkinson’s

disease and Huntingdon's disease, both of which are linked to impaired striatal function (Dubois, Defontaines et al. 1995; Saint-Cyr, Taylor et al. 1995). Accounting for the role of the striatum in both initiation and suppression of movements, it would be plausible to suggest a role for the striatum in response inhibition. Animal studies using bilateral excitotoxic lesions of the nucleus accumbens core, but not shell, increase impulsive choice for small immediate rewards (Cardinal, Pennicott et al. 2001; Pothuizen, Jongen-Relo et al. 2005). Lesions to the medial striatum have also been reported to increase motor impulsivity in the 5CSRTT (Rogers, Baunez et al. 2001). However lesions to the rat nucleus accumbens shell and core combined decrease impulsive choice (Acheson, Farrar et al. 2006) but increase impulsive action in the 5CSRTT (Christakou, Robbins et al. 2004) which indicate dissociable roles of the striatum in impulsivity. It has also been reported that excitotoxic lesions to the basolateral amygdala, a brain area with strong connections to the nucleus accumbens, leads to increased impulsive choice (Winstanley, Theobald et al. 2004). This collated evidence may imply a relationship for the striatum and the PFC in modulating impulsivity, and furthermore, that the basolateral amygdala and the nucleus accumbens may interact to regulate different types of impulsive behaviour.

1.12 Behavioural monitoring:

Behavioural monitoring i.e. the ability to monitor one's ongoing performance is of critical importance to behavioural control. The ability to detect an error serves an adaptive function in signalling to an individual that a change in behaviour might be more advantageous (MacDonald, Cohen et al. 2000; Botvinick, Braver et al. 2001). Failure to detect or appreciate the importance of errors has been shown to correlate with many clinical symptoms including loss of insight (Ott, Lafleche et al. 1996) and perseverative behaviour (Liddle, Friston et al. 1992; Liddle, Kiehl et al. 2001). The anterior cingulate cortex has been implicated in error-related function (Dehaene 1994) and error-related hypoactivity has been observed in cocaine users (Kaufman, Ross et al. 2003), and opiate users (Forman, Dougherty et al. 2004; Lee, Zhou et al. 2005; Yucel and Lubman 2007). In a Stroop task, error-related hypoactivity has also been observed in cocaine users (Bolla, Ernst et al. 2004), cannabis users (Eldreth, Matochik et al. 2004) and following alcohol administration (Ridderinkhof, de Vlugt et al. 2002).

These findings suggest this phenomenon may be common to drug abuse *per se* rather than attributable to the different mechanisms of action of each drug.

The anterior cingulate cortex's role in error detection may be driven by the same mesocorticolimbic dopamine system that involves the nucleus accumbens in the reinforcing effects of cocaine (Holroyd and Coles 2002). A functional deficit in the anterior cingulate cortex may contribute considerably to the addiction cycle as recent theorists have proposed that the anterior cingulate cortex monitors not errors *per se*, but the likelihood of errors (Brown and Braver 2005; Magno, Foxe et al. 2006); hence the anterior cingulate cortex assesses risk and uncertainty in decision making (Paulus and Frank 2006). Anterior cingulate dysfunction may result in a higher likelihood of risky decision making concerning drug use and the inability to detect the likelihood of drug increases. Studies conducted by Hester et al (Hester, Simoes-Franklin et al. 2007) using a modified version of the go / no go task to measure post-error slowing, an adaptive response to improve performance following an error commission and error awareness, to test their own error detection, revealed that although cocaine users did not differ from control in their post- error slowing, they detected fewer of their own errors. This finding suggests that drug use / abuse is correlated with anterior cingulate cortex functional hypoactivity, and we might postulate that drug dependence, including alcoholism, result in compromised error detection which contributes to a higher likelihood of risky decisions which may perpetuate drug use / abuse.

1.13 Summary:

In recent years, a vast array of data has been published implicating a role for the prefrontal cortex in drug abuse, with particular regard to executive functions. However, the exact role that the PFC plays in addiction in behavioural indices remains unclear. The prefrontal brain circuits in an addicted brain is significantly and functionally altered in comparison to a non-addicted brain, evidence for which is derived from brain imaging studies using positron emission tomography and functional magnetic resonance imaging. Volkow and associates proposed that drug addiction disrupts the function of 4 brain circuits, one of which is located in the prefrontal cortex, namely the control circuit. It is thought that dysfunction of the control circuit resulting from drug abuse leads to a loss of control of this brain circuit,

which results in less influence of this control circuit in the PFC on the reward, motivation and learning and memory circuits. The result of this loss of control may be impairment of inhibitory control and poor decision making, which perpetuates the addiction cycle.

Although, function of the prefrontal cortex may be measured in humans using neuropsychological tasks, PFC-related behaviours are particularly challenging to measure in animal models, as the PFC is responsible for higher order processing. At present the tests for prefrontal cortical function attempt to measure executive functions such attentional control, inhibitory control and behavioural monitoring in humans. Attentional control measured using the Stroop task in which irrelevant distracting information will slow responses and reduce accuracy. Inhibitory control is the ability of an individual to suppress interfering stimuli in order to attend selectively to certain information in a complex environment (Dagenbach 1994). Poor response inhibition has been postulated to result in elevated impulsivity, a plausible suggestion which is derived from the role of the PFC in impulse control; hence, if an individual suffers from poor impulse control, there is a higher likelihood of that individual exhibiting higher impulsivity levels. Behavioural monitoring involves the ability to monitor one's ongoing performance and ability to detect errors with a view to change strategies to improve performance (Botvinick, Braver et al. 2001). In animal models, PFC function may be extrapolated from indices of impulsivity (as measured by delay discounting which measures impulsive choice, go / no go task (Newman, Widom et al. 1985), the stop signal reaction time task (Logan, Cowan et al. 1984) and the 5 choice serial reaction time task (Carli, Robbins et al. 1983; Robbins 2002).

Repeated ethanol withdrawal gives rise to perseverative responding in rats on a fixed interval operant task (Borlikova, Elbers et al. 2006) and response disinhibition in the form of resistance to extinction of conditioned fear (Ripley, O'Shea et al. 2003) and in humans diminished ability to withhold a prepotent response as measured by the vigilance task in the Gordon Diagnostic system in female bingers in comparison to male bingers (Townshend and Duka 2005). These findings implicate the control circuit as proposed by Volkow and associates (Volkow, Fowler et al. 2003) and point towards dysfunction of PFC and anterior cingulate gyrus. Neither Borlikova (Borlikova, Elbers et

al. 2006) or Ripley's (Ripley, O'Shea et al. 2003) investigations in rats measured response inhibition directly in relation to withholding a prepotent response. Hence in this current thesis, a novel 2 choice serial reaction time task was devised – modelled on the principles of the well established 5 choice serial reaction time task - to measure indices of attention and motor impulsivity.

The present rat model of repeated ethanol withdrawal has provided several behavioural parallels with human alcohol detoxification. However, the method is not well described in terms of other measures. For this reason, behavioural measures of withdrawal severity will be investigated, including measurements of sleep architecture, home cage locomotor activity, core body temperature and post withdrawal food and water intake as a function of single or repeated ethanol withdrawal, to characterise patterns of ethanol taking, and withdrawal consequences. From rat studies conducted by Spanagel and associates (Spanagel, Putzke et al. 1996) ethanol withdrawal has been reported to reduce post withdrawal food intake, hyperthermia and hyperlocomotion. Sleep architecture disruptions have also been reported in a mouse model of repeated ethanol withdrawal (Veatch 2006) by disruptions in total time asleep across the acute withdrawal period, a reduction in NREM sleep accompanied by a concomitant increase in REM sleep which lasted for at least 3 days after withdrawal. Hence it would be plausible to suggest that our model of repeated ethanol withdrawal would reflect the findings reported by both Spanagel (Spanagel, Putzke et al. 1996) and Veatch (Veatch 2006).

1.14 Aims and Structure of thesis:

Chapter 2:

Chapter 2 investigates behavioural measures of alcohol withdrawal severity. Spanagel and colleagues reported withdrawal symptoms of hyperthermia, hyperlocomotion, transiently enhanced food intake which decreased after the first day of cessation of forced alcohol drinking (Spanagel, Putzke et al. 1996). Considering the physical signs of alcohol withdrawal have been reported by Hunter et al (Hunter, Riley et al. 1975) and Majchrowicz and associates (Majchrowicz 1975) have used subjective ratings of severe physical alcohol withdrawal symptoms, it was difficult to compare withdrawal severity

across laboratories. Further complications are involved in establishing the timings at which rats experience withdrawal, particularly the acute stages of withdrawal. Therefore, a fully automated radiotelemetric system was employed to observe changes in core body temperature, activity and sleep architecture. This system allowed continuous monitoring and quantitative assessment of withdrawal-induced behaviours which would otherwise be impossible if behaviours were analysed using subjective observational analysis. Furthermore, post-withdrawal food and water intake was measured as an index of withdrawal severity. This chapter should highlight the physiological changes that occur as a result of our well-established rat model of repeated ethanol withdrawal and further investigate how our rat model affects the aforementioned physiological withdrawal symptoms.

Chapter 3:

Chapter 3 uses the expression of immediate early genes c-*Fos,* a molecular marker for neuronal activation and zif268, a marker for neuronal plasticity, to further investigate brain activation and plasticity as a consequence of repeated episodes of ethanol withdrawal in the prefrontal cortex. It has been reported that the frontal lobes are particularly sensitive to chronic ethanol consumption (Moselhy, Georgiou et al. 2001) which may result in subsequent cognitive impairments observed in human alcoholics and binge drinkers (Duka, Townshend et al. 2003; Townshend and Duka 2003; Townshend and Duka 2005; Scaife and Duka 2009). Hence, the aim of this chapter is to investigate prefrontal brain activation at a molecular level and thus allow for consideration for further experimentation of behavioural measures.

Chapter 4:

The prefrontal cortex is responsible for executive functions including impulse control. Hence PFC dysfunction may lead to impairments in impulse control resulting in elevated impulsivity levels (Evenden 1999). Studies investigating the effects of repeated ethanol withdrawal on fixed interval responding and fear conditioning reveal that multiply withdrawn rats exhibit over-responding on a fixed interval schedule and resistance to extinction of fear conditioning, implicating a role for both the amygdala and the PFC.

By devising a novel 2 choice serial reaction time task to capture behavioural measures of attention and impulsivity, this chapter aims to further investigate the effects of repeated ethanol withdrawal on impulsivity and attention levels.

Chapter 5:

The PFC also plays an important role in maintaining and shifting attentional set. Chapter 5 investigates the effects of repeated ethanol withdrawal on attentional set shifting using the intradimensional extradimensional set shifting task, first devised by Lawrence (Lawrence 1949) and Berg (Berg 1948) and refined by Birrell and Brown (Birrell and Brown 2000).

Chapter 2

The effects of repeated ethanol withdrawal on withdrawal severity

2.1 Introduction:

2.1.1 Alcohol withdrawal Syndrome:

The alcohol withdrawal syndrome comprises a number of symptoms associated with the sudden cessation of alcohol consumption after prolonged use / abuse. These symptoms can range from mild or moderate physical and psychological symptoms, such as sweating, nausea, loss of appetite, insomnia, nervousness, anxiety, irritability, mood swings, depression, to severe symptoms that include delirium tremens, hallucinations, fever and convulsions (Hershon 1977). All these symptoms can be attributed to the neuroadaptive changes which occur as a result of heavy chronic ethanol use or abuse (Koob and Le Moal 1997; Nutt 1999).

Neuroadaptations occur within the brain during the development of alcohol dependence, which allow the brain to function under conditions of excessive ethanol consumption. Heavy ethanol consumption reduces excitatory processes of the central nervous system (CNS) while simultaneously enhancing the inhibitory processes (Dodd 1996; Buckley, Eckert et al. 2000; Devaud and Alele 2004; Alele and Devaud 2005; Taylor, Nash et al. 2008). Alcohol withdrawal can result in CNS hyperexcitation due to an altered homeostatic set point which consequently leads to the aforementioned physical withdrawal symptoms. In withdrawal, the hyperexcited brain state is thought to be attributable to an increase in glutamate excitatory transmission (Grant, Valverius et al. 1990; Hoffman, Rabe et al. 1990; Hoffman, Grant et al. 1992). There is considerable evidence that glutamate-mediated excitatory neurotransmission plays a crucial role in mediating the behavioural actions of prolonged ethanol exposure that underlies ethanol dependence (Grant and Lovinger 1995; Eckardt, File et al. 1998; Krystal 2002; Krystal, Petrakis et al. 2003). An in vitro study by Roberto (Roberto, Schweitzer et al. 2004) reported that chronic ethanol treatment sensitised NMDA

receptors, a subtype of the ionotropic glutamate receptor family, indicating that chronic ethanol treatment led to neuroadaptations in glutamatergic neurotransmission, particularly via NMDA receptors which may play an important role in ethanol dependence (Kalluri, Mehta et al. 1998; Darstein, Landwehrmeyer et al. 2000; Narita, Soma et al. 2000). This chronic ethanol effect is additionally compounded by the effect of reduced $GABA_A$ inhibitory transmission (Roberto, Cruz et al.; Nutt and Peters 1994; Roberto, Schweitzer et al. 2004; Roberto, Bajo et al. 2006; Kumar, Porcu et al. 2009). It is thought that the altered balance between the excitatory effects of glutamate and the inhibitory effects of GABA when alcohol consumption is discontinued gives rise to the alcohol withdrawal syndrome.

2.1.2 Measures of Ethanol Withdrawal Severity:

The severity of the withdrawal symptoms may lead the alcoholic to relapse back to alcohol drinking, hence continuing the cycle of addictive behaviour (Cooney, Litt et al. 1997; Koob, Roberts et al. 1998; Sinha and O'Malley 1999; Roberts, Heyser et al. 2000; Sinha 2001; Willinger, Lenzinger et al. 2002; Griffin, Lopez et al. 2009). The higher the number of detoxifications an alcoholic experiences, the greater the CNS hyperactivation, contributing to increased severity of subsequent ethanol withdrawals (Ballenger and Post 1978; Baker and Cannon 1979; Brown, Anton et al. 1988; Lechtenberg and Worner 1991; Booth and Blow 1993).

Brown at al (1988) reported that alcoholic patients (48%) who experienced 5 or more previous withdrawal episodes were more susceptible to withdrawal-related seizures during detoxification compared with 12% of alcoholics with a similar withdrawal history (Brown, Anton et al. 1988). This correlation provides support for the hypothesis that the more detoxifications experienced by the alcoholic, the greater the incidence and severity of withdrawal-induced seizures. In their review, Ballenger and Post (1978) proposed that alcohol withdrawal symptoms became increasingly more severe by a process similar to kindling. Kindling, a phenomenon first described in epilepsy research, refers to the phenomenon by which small electrical stimulations (producing excitation) in specific brain areas result in a progressively greater effect on repeated application using the same level of stimulation. The initial electrical

stimulation was subthreshold in that it did not produce sufficient brain activation and did not result in convulsions. However, after several repeated episodes, the same stimulation now elicited an epileptic seizure. In multiply withdrawn mice, the seizure threshold was reported to be progressively lowered with every detoxification (Becker 1996); hence in human alcoholics, life-threatening convulsions may possibly be attributed to CNS excitation arising from previous experience of ethanol withdrawal.

From the findings of previous studies conducted in this laboratory which have found evidence for kindling-like processes occurring as a consequence of repeated ethanol withdrawal (Stephens, Brown et al. 2001; Ripley, Dunworth et al. 2002; Ripley, Brown et al. 2003), we propose the process of ethanol withdrawal acted as a sub-threshold stimulation on specific brain areas, and following repeated episodes of withdrawal the brain became sensitised, leading to greater behavioural effects than during the initial withdrawal.

Other symptoms of acute ethanol withdrawal, such as anxiety have also been reported to progressively increase as a result of repeated withdrawals in human studies (Malcolm, Myrick et al. 2002) and some animal models (Holter, Engelmann et al. 1998; Overstreet, Knapp et al. 2002; Breese, Overstreet et al. 2005). On the contrary, a clinical study by Duka et al (2002) found although anxiety levels were increased in patients undergoing detoxification, this was not influenced by previous experience of detoxification (Duka, Townshend et al. 2002). This finding was supported by animal studies which reported increased anxiety-like behaviour in the elevated plus maze in rats that had experienced both single and repeated ethanol withdrawal; however no difference was found between the alcohol-treated groups (Borlikova, Le Merrer et al. 2006). Anxiety can be tested in animal models using the elevated plus maze which comprises a cross-shaped apparatus, raised approximately 1 metre from the floor. Two arms of the four are enclosed and the other two arms are open. An anxious rat will spend significantly more time in the enclosed arms whereas low anxiety is reflected by more time spent in the open arms. Studies by other laboratories found a single alcohol withdrawal resulted in a higher level of anxiety, as observed in the increased amount of time a rat spent in the enclosed arms of the plus maze (File, Andrews et al. 1993; Wilson, Watson et al. 1998; Gatch, Wallis et al. 1999). Anxiety is, in part, mediated by

the amygdala, a component of the limbic system within the brain and studies by Borlikova et al have found increases in the expression of immediate early gene c-*fos* in the amygdala as a result of repeated ethanol withdrawal (Borlikova, Le Merrer et al. 2006). Furthermore, in conditioned emotional response tasks, alcohol-fed rats showed less sensitivity to increases in shock level, in particular the RWD rats, which showed a reduced ability to acquire a conditioned emotional response to a footshock; although SWD rats also showed a reduced ability to acquire a conditioned emotional response, this was not as profound as observed in the RWD rats (Stephens, Brown et al. 2001). Impaired conditioned emotional response in repeatedly withdrawn rats was additionally supported by electrophysiological studies revealing that repeated ethanol withdrawal led to a reduction in long-term potentiation in limbic brain areas, suggesting that repeated ethanol withdrawal reduces synaptic plasticity which in turn reduced the capacity for future learning, with respect to fear conditioning (Stephens, Ripley et al. 2005). These combined findings indicate that ethanol withdrawal, regardless of whether the experience is single or repeated, lead to long-term changes in the limbic brain circuitry which may result in behavioural changes in anxiety. It is unclear whether increased anxiety during withdrawal episodes results in increased susceptibility of an alcoholic to relapse.

Studies investigating the early stages of ethanol withdrawal found that locomotor activity and body temperature were significantly increased 18 h after withdrawal (Spanagel, Putzke et al. 1996). Spanagel and colleagues investigated post-withdrawal food and water intakes and reported that initially, food intakes were enhanced, but, one day after withdrawal, food intake was significantly reduced compared with control rats. An initial significant increase in water intakes was also observed in alcohol rats compared with control rats, which lasted 12 h. The authors also observed hyperlocomotion and hyperthermia in the alcohol-fed rats, both of which are behavioural changes commonly associated with the ethanol withdrawal syndrome. In this present study, we investigate whether these findings can be observed in our rat model of repeated ethanol withdrawal.

2.1.3 Sleep Disturbances as a Measure of Withdrawal Severity:

There is evidence in both human and animal studies that link alcohol dependence with disruptions in sleep patterns and architecture (Clark, Gillin et al. 1998; Drummond, Gillin et al. 1998; Kubota, De et al. 2002; Veatch 2006). Sleep problems in alcoholics during detoxification, and in abstinent alcoholics are a major problem as the disturbances may contribute to an increased rate of relapse to alcohol abuse. For instance, insomnia occurs in 36-72% of alcoholic patients which may last for weeks to months after abstinence from alcohol (Brower 2003). The co-occurrence of insomnia and alcoholism is of significant clinical importance as alcoholism may exacerbate insomnia, the consequences of which can include impaired daytime performance (Ancoli-Israel and Roth 1999; Roth and Ancoli-Israel 1999), memory dysfunction (Roehrs and Roth 2001) and increased risk for depression (Gillin, Smith et al. 1990; Gillin, Smith et al. 1994; Weissman, Greenwald et al. 1997; Clark, Gillin et al. 1998; Drummond, Gillin et al. 1998). Insomnia experienced by patients entering treatment for alcoholism has been significantly correlated with subsequent alcoholic relapse. Although it remains unclear whether treatment for insomnia in alcoholic patients reduces relapse rates, the association between the two factors may be of major importance for future clinical treatment.

Sleep disturbances may often continue beyond early ethanol withdrawal, suggesting that ethanol dependence and withdrawal result in long-term changes in the brain areas responsible for sleep. Williams (Williams and Rundell 1981) found that recently abstinent chronic alcoholics exhibit “fractured” sleep, in which sleep onset is delayed and the rhythmic pattern of sleep is disrupted.

2.1.4 Sleep Architecture as determined by EEG / EMG:

Sleep architecture is composed of 2 main states, REM, which is dominated by rapid eye movement and EEG activity in the theta bandwidth together with profound loss of muscle tone, and non-REM, which is devoid of rapid eye movement and shows activity in the sigma and delta bandwidths. There is dynamic switching between the two sleep states and wakefulness. In this current study, sleep was measured by 2 simultaneous electrophysiological measurements.

An electroencephalogram (EEG) is a measurement which traces brain electrical activity through electrodes placed on the scalp. EEG measurements produce characteristic brain waves called alpha, beta, delta and theta rhythms which can be differentiated by their frequencies whereas an electromyogram (EMG) measures the electrical activity of muscles through electrodes placed on the skin in various body regions. Electromyograms can be used to measure electrical activity in muscles underlying small movement such as twitching during sleep. Electromyograms provide information about muscle activity whereas an electroencephalogram differentiates between NREM and REM sleep from which sleep architecture can be determined. The EEG recording during REM and WAKE states are similar, hence EMG provides further information to distinguish between REM sleep and the WAKE state of the rat.

As disrupted sleep patterns are a hallmark of alcohol withdrawal, it was our interest to investigate whether our well-established model of repeated ethanol withdrawal produced a similar disruption of sleep architecture in the rat, during and following chronic ethanol treatment and withdrawal. Based on studies of repeated cycles of chronic ethanol exposure on sleep architecture in mice and rats, we would expect to see similar sleep architecture disruption in our rat model of repeated ethanol withdrawal.

2.1.5 Experimental Aims:

This current study investigated the effects of repeated ethanol withdrawal on severity of withdrawal. A radio-telemetric system (available at Pfizer Global Research and Development, UK) was used to monitor EEG /EMG measures, core body temperature and activity for the duration of chronic ethanol treatment and beyond the final withdrawal. In a separate experiment conducted at University of Sussex, food and water intakes were measured during the post withdrawal period for 4-5 days. Blood ethanol levels from tail vein samples were measured to observe the correlation between the volume of liquid diet consumed based on daily weight measurement of the liquid diet bottles and the amount of ethanol detectable in the bloodstream. Based on studies of repeated cycles of chronic ethanol exposure on severity of withdrawal

conducted using mice and rats, we expected to observe increased severity of withdrawal in our rat model of repeated ethanol withdrawal.

2.2 Materials and Methods:

2.2.1 Chronic ethanol liquid diet treatment:

Rats were randomly allocated into three treatment groups; control group (CON), single withdrawal (SWD) group and repeated ethanol withdrawal (RWD) group, n=8. The SWD and RWD groups were fed a nutritionally complete 7% ethanol-containing liquid diet (Dyets, Bethlehem, PA, USA) as their sole food source. The CON group were fed a calorifically equivalent control liquid diet (Dyets, Bethlehem, PA, USA) for 24 days. The SWD group were fed the ethanol liquid diet for 24 days. Both SWD and RWD groups were given an excess quantity of ethanol-containing liquid diet (on the treatment days that they received ethanol-liquid diet i.e. not on withdrawal days) to allow as much ethanol exposure as possible. The CON group were given a restricted quantity of control liquid diet, calculated by the mean amount of ethanol-containing liquid diet consumed by the SWD and RWD groups on the previous experimental day, which ensured that the CON consumed an equal quantity of liquid diet compared with SWD and RWD groups.

The RWD group received liquid diet for a total of 30 days and experienced two intermediate withdrawals lasting 3 days each (experimental days 11 – 13 and 21 – 23.) During the intermediate withdrawals, the ethanol liquid diet was removed and the RWD group was given control liquid diet which was restricted to the mean amount of diet consumed by the ethanol-fed rats the previous day. Rats received fresh liquid diet daily. Ethanol consumption was calculated as grams of ethanol consumed per kg of body weight. The RWD group had the same level of ethanol exposure as the SWD group, which accounted for the additional withdrawal days experienced by the RWD group. On the final treatment day, all animals were withdrawn from liquid diet at 08:00 h and remained in their home cages. They were given *ad libitum* access to fresh water and rat chow.

2.2.2 Experiment 1: Telemetry measurements of EEG / EMG, activity and body temperature and Blood ethanol concentrations.

Subjects:

Twenty four Lister hooded rats (Charles River, UK) were used to measure core body temperature, activity and EEG / EMG. Data were collected using an automated radio-telemetric system. Rats weighed approximately 175g at the beginning of the experiment, prior to undergoing surgery. Experiments using the radio-telemetry system were conducted at Pfizer Global Research and Development, as part of the Drug Safety Research and Development Department. At the beginning of the experiment, rats were group-housed in fours, and maintained on *ad libitum* standard rat chow and water under controlled room conditions of 21±2°C temperature, 50±10% humidity, in a 12/12 hr light/dark cycle (lights on at 07:00h). Rats were acclimatised to the home cage for 7 days prior to undergoing surgery for the implantation of radiotransmitters into the intraperitoneal cavity. All experiments were conducted under the UK Animal (Scientific Procedures) Act, 1986 following Home Office approval.

2.2.3 Blood sampling procedure:

Blood samples (250 µL) were collected from the tail vein of each rat in SWD and RWD groups at various stages during chronic ethanol treatment to determine blood ethanol concentrations. No more than 7% of the total blood volume of each rat was collected over the period of ethanol treatment, in accordance with Home Office guidelines. This experiment was conducted at Pfizer Global Research and Development, Sandwich, UK.

In the SWD group, blood samples were taken on days 8, 11, 20, 22 and 24. The RWD group had blood samples collected on days 10, 11, 14, 17, 18, 20, 22 and 24. Tail vein blood samples were collected on each specified day at approximately 09:30h; approximately 1 h after fresh ethanol liquid diet was administered. Each 250 µL blood sample was collected using a 25g (blue) needle into a 1ml polypropylene screw cap tube with lithium heparin bead. Samples were immediately centrifuged for 10 minutes and the top layer of plasma was drawn from the sample tubes, separated into aliquots which were stored at -80°C to prevent the ethanol from evaporating from the samples.

Blood ethanol concentrations were calculated by oxidising ethanol (using the co-enzyme NAD^+) to acetaldehyde in the presence of ethanol dehydrogenase.

$$\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ethanol dehydrogenase}} \text{acetaldehyde} + \text{NADH analogue}$$

The apparatus (Bayer® Clinical method for Advia 1650) measured the rate of increase in absorbance at 340 nm due to the reduction of co-enzyme NAD^+ analogue to NADH analogue, with corresponds to the amount of ethanol in the sample.

2.2.4 Implantation of radio-transmitters:

All surgical procedures were conducted by the Animal Surgery team at Pfizer Ltd, based at Sandwich. Radiotransmitters were surgically implanted into the intraperitoneal cavity in order to measure sleep, activity and core body temperature. On the day prior to surgery, rats were dosed with Baytril treatment (Carprofen) in their drinking water (dose: 0.1ml/100g body weight s.c. of a 1:10 dilution of the supplied 50mg/ml solution) which continued for 5 days post-surgery. Rats were anaesthetised with isoflurane (5% in O_2) administered in an induction chamber. Anaesthesia was maintained via a semi-open nose cone (isoflurane 2.5%). Surgery was performed for intraperitoneal implantation of telemetry probes under general anaesthesia by the Pfizer surgery team. The bio-potential leads were positioned and sutured subcutaneously for the measurement of EEG. EEG electrodes were placed in a fronto-occipital position (2 mm lateral and 2 mm anterior to bregma, and 2 mm lateral and 2 mm anterior to lambda). EMG leads were implanted in the neck muscles (one on each side). Placement of the implant in the peritoneum allowed measurement of intraperitoneal temperature. Directly after implantation, rats were transferred to a warm air chamber for 10 mins to recover from anaesthesia. Rats were singly housed directly into home cages post surgery and received a wet mash diet. After rats were certified fit for use on the study by the named veterinary surgeon at Pfizer Global Research and Development, they were given 3 days to acclimatise to their home cages before commencement of radio-telemetry recordings.

Baseline levels of activity were recorded for 2 days prior to administration of liquid diet using telemetry receivers. The data receivers which measured the signal from the radio-transmitters were placed on benches and home cages were placed on the telemetry data receivers. Data receivers measured the change in signal strength acquired from the implanted telemetry transducers. This signal, recorded via the Data Sciences International system was also used to infer spontaneous activity. Data were captured, stored and analysed in real time by a PC based digital acquisition system using Notocord software. Telemetry data were recorded in 22 hour block files. After baseline activity was established, rats were administered the liquid diet treatment (see chronic ethanol treatment). Data for EEG / EMG, activity and body temperature were collected simultaneously on a daily basis.

2.2.5 Recording and Analysis:

EEG and EMG data were captured continuously with Data Sciences International hardware and Data Acquisition Gold Software on specified treatment days during chronic ethanol or control liquid diet administration (see Appendix 2.15). Recording of EEG and EMG signals began at 09:00h on each treatment day and was collected for 22 hours. The recorded file of each specified treatment day was then imported into Spike2 (Cambridge Electronics Ltd, Cambridge, UK) for further analysis.

Vigilance states were automatically scored with software developed in house by Dr Magnus Ivarsson at Pfizer Ltd. The sleep stage discriminator used logic based on changes of EEG and EMG activity that defined the different vigilance states in the rat as outlined by Ivarsson et al (Ivarsson, Paterson et al. 2005).

NREM states exhibit synchronised, high amplitude EEG activity accompanied by low muscle activity, REM shows desynchronised low amplitude EEG activity with low or absent EMG activity. Artefacts were removed automatically using software developed in-house by Dr Iverrson. After the removal of 12-second epochs with artefacts, in-house software used the relationship between the rectified and integrated signal of EEG and EMG traces to assign each epoch to one of the 3 vigilance states; WAKE, REM and NREM. If the rectified and integrated EEG signal in a single epoch was larger than the average rectified and integrated signal of all artefact-free epochs over 12 hours

multiplied by a constant (*k*) for each individual rat, the epoch was labelled "large" and if the epoch was smaller than the average, the epoch was labelled "small." For each epoch, if the EEG was determined to be "large" and the corresponding EMG was determined to be "small", the epoch in question would be classified as NREM. For REM classifications, both EMG and EEG recordings in the epoch would be determined as "small." For WAKE classification in an epoch, the EEG would be "small" and the EMG would be "large."

2.2.6 Experiment 2: Post withdrawal food and water intake

Subjects:

Eighteen male Lister hooded rats (Harlan, UK) weighing 505 g ± 8.92 at the beginning of the experiment were pair-housed and maintained on *ad libitum* standard rat chow and water under controlled room conditions of 21±2°C temperature, 50±10% humidity, in a 12/12 hr light/dark cycle (lights on at 07:00h). All experiments were conducted under the UK Animal (Scientific Procedures) Act, 1986 following Home Office approval. This experiment was conducted at University of Sussex Animal Facilities Unit.

2.2.7 Procedure:

Rats were administered either control liquid diet or 7% ethanol containing liquid diet as described previously in this chapter (section 2.2.1). On the final withdrawal day, rats had their liquid diet bottles removed from their home cages at 09:00 h. Food hoppers were filled with rat chow and weighed prior to allowing rats *ad libitum* access to rat chow. Water bottles were filled with fresh water and weighed before being given to rats. At 09:00h on the following day, the food hoppers and water bottles were re-weighed to determine the amount of food and water consumed over a 24 hour period. This procedure was conducted over 4 – 5 days post withdrawal.

2.2.8 Data Analysis:

Analysis was conducted using SPSS version 16.0. Statistical analysis of WAKE, REM and NREM sleep differences between the 3 treatment groups were analysed using repeated measures analysis of variance (ANOVAs) with treatment groups serving as a between-subject variable and treatment day (baseline and day 8) and time bin serving

as a repeating measure (11am-1pm, 1pm-4pm, 4pm-7pm, 7pm-10pm, 10pm-1am, 1am – 4am, 4am-6am). Time bins 11am-1pm and 4am-6am were 2 hours in length which in comparison to 3 hour time bins, were at the beginning and end of the recording period. The 2 hour time bin at 11am-1pm accounted for the instability in data recording at the beginning of data acquisition (at 9am-11am) due to blood sampling and administration of fresh liquid diet. The 2 hour time bin at 4am-6am accounted for instability of data acquisition from 7am onwards. Adjusted Greenhouse-Geisser probabilities with Bonferroni corrections were used as appropriate in which case epsilon values were quoted. Post hoc comparisons were conducted as appropriate. For the data collected on days 11, 18, 24 and post-withdrawal day 3, planned comparisons were used to compare group x time bin effects.

2.3 Results:

2.3.1 Experiment 1: Chronic ethanol Consumption

As can be observed in **Figure 2.2,** there were no significant differences in ethanol consumption between SWD and RWD groups (p = 0.3). Ethanol consumption was stable across the period of chronic ethanol treatment for both SWD and RWD groups. The comparison between the amount of ethanol consumed **(figure 2.2)** and blood ethanol levels **(figure 2.4)** showed the difference between the two measurements. Rats consumed a high and stable level of ethanol liquid diet.

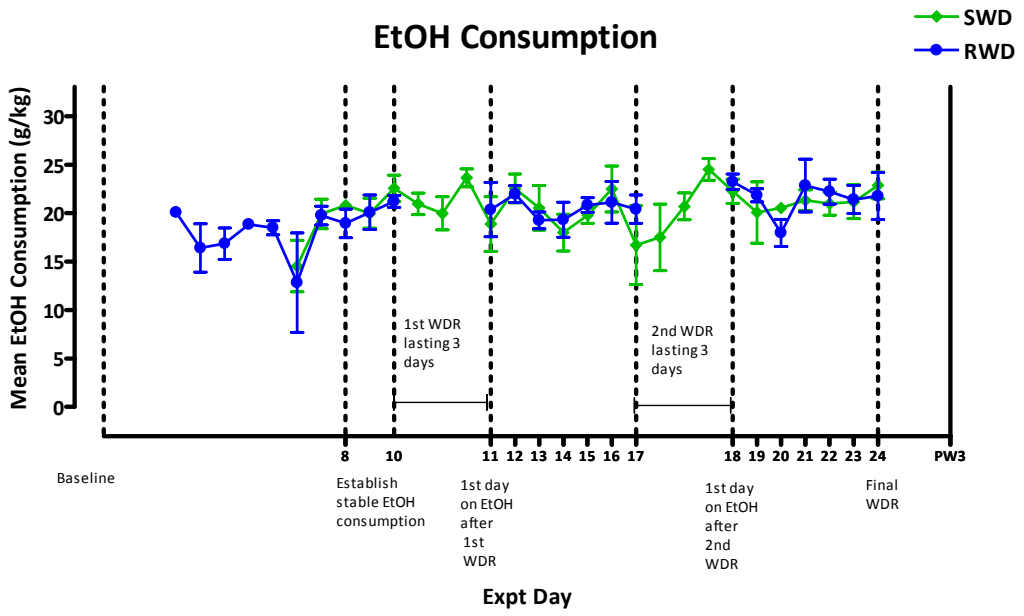


Fig 2.2: Ethanol consumption expressed as g per kg of body weight per experimental day. Data represented mean ± sem. Arrow indicated the final withdrawal; intermediate withdrawal episodes in RWD group took place on experimental days 11 – 13 and 21 – 23. There was no overall difference between the groups in consumption (p = 0.3). SWD and RWD groups consumed an average of 21 g/kg.

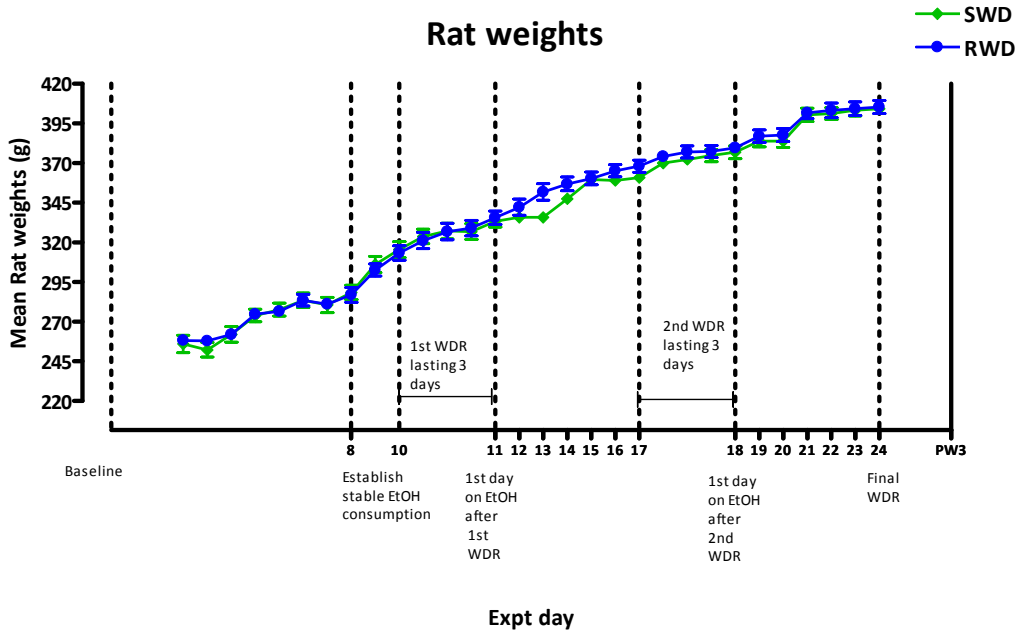


Fig 2.3: Rat weights as measured for each experimental day. Data represented mean ± sem.

2.3.2 Blood ethanol levels during chronic ethanol treatment:

Figure 2.4 illustrates the blood ethanol levels throughout the time course of chronic ethanol treatment. Planned comparisons were made between the SWD and RWD groups on days 8/10, 11, 20, 22 and day 24 (final withdrawal day). There was no significant group difference in blood ethanol levels on day 8/10 (p = 0.11), day 11 (p = 0.14), day 20 (p = 0.96), day 22 (p = 0.72) and day 24 (p = 0.49). Hence, there were no significant differences in BECs (Blood ethanol concentrations) between SWD and RWD groups during the course of chronic ethanol treatment.

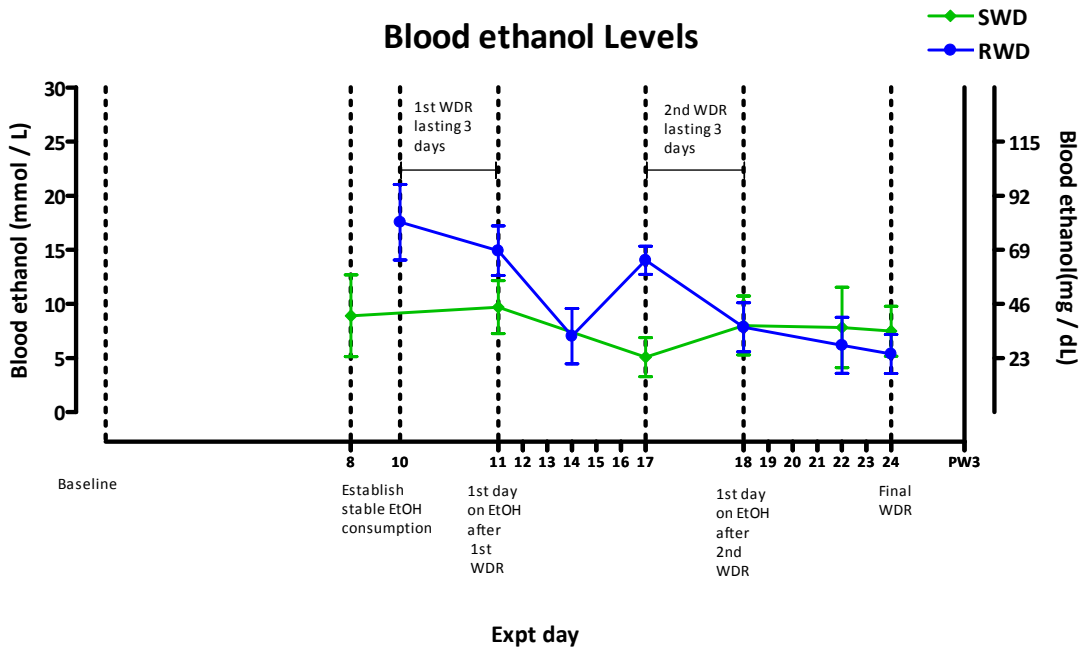


Fig.2.4: Blood ethanol levels of SWD and RWD animals during chronic ethanol treatment and post withdrawal. Data represented mean blood ethanol ± sem. Tail vein samples were taken at various stages during chronic ethanol treatment. Blood sample collection was conducted on each collection day at approximately 09:30h. Black dotted lines represent the 2 intermediate withdrawal periods for RWD group.

2.4 EEG / EMG Measurements of Sleep states:

Analysis of time bins excluded the time bin 9am-10am in order to account for disturbances to EEG / EMG patterns due to experimental procedures. Data were analysed using a two-way ANOVA and planned comparisons.

2.4.1 Baseline WAKE:

As depicted by **Figure 2.5a**, wakefulness increased from 4pm-10pm and then remained at relatively high levels throughout the dark phase, as expected of rodent circadian rhythms. The highest level of wakefulness was found during 7pm-10pm during the dark phase at baseline.

A repeated measures ANOVA revealed a significant main effect of time bin on WAKE ($F_{6, 108}$ = 39.3, $p < 0.001$), further analysis of data revealed that highest levels of wakefulness was found during 7pm-10pm during the dark phase. A significant group x time bin interaction was found ($F_{12, 108}$ = 4.46, $p = 0.001$) which indicates that the treatment groups behaved differently as a function of time. However, no significant group differences were found ($F_{2, 18}$ = 0.62, $p = 0.55$).

2.4.2 Baseline NREM:

As depicted by **Figure 2.5b**, high levels of NREM sleep were observed during the light phase and NREM sleep decreased from 4pm-10pm and then remained at relatively steady levels throughout the dark phase, as expected of rodent circadian rhythms. The highest level of NREM sleep was found during 1pm – 4pm during the light phase at baseline.

Statistical analysis using a repeated measures ANOVA found a significant main effect of time bin on NREM ($F_{6, 108}$ = 41.8, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 108}$ = 4.99, $p < 0.001$) which indicates that the treatment groups behaved differently as a function of time. However, no significant group differences were found ($F_{2, 18}$ = 0.314, p = 0.734).

2.4.3 Baseline REM:

As demonstrated by **Figure 2.5c**, observed levels of REM sleep remained at relatively stable low levels throughout the course of the day.

A repeated measures ANOVA found no significant main effect of time bin on REM was found ($F_{6, 102}$ = 1.7, $p = 0.18$), indicating that measures of REM were not affected across the day. No significant group x time bin interaction was found ($F_{12, 102}$ = 1.15, $p = 0.35$). Furthermore, no significant group differences were found ($F_{2, 17}$ = 0.4, $p = 0.68$).

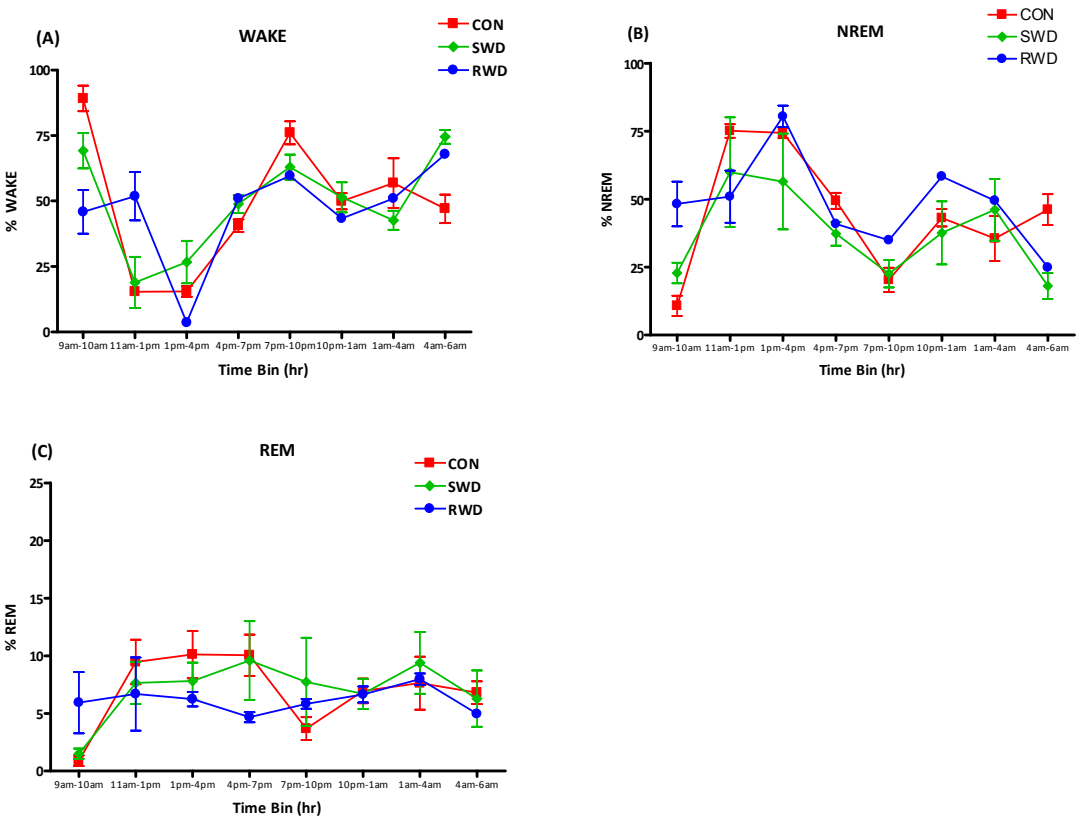


Figure 2.5: Mean baseline measures of (A) % wake, (B) % NREM and (C) % REM sleep as measured by EEG / EMG recordings.

2.4.4 Day 8 WAKE:

As depicted by **Figure 2.6a**, the effects of ethanol decreased wakefulness from 11am-10pm, after which time chronic ethanol treatment appeared to increased wakefulness from 10pm – 6am. Control rats showed a peak in wakefulness at 7pm-10pm which was consistent with expected rodent circadian rhythms.

A repeated measures ANOVA revealed a significant main effect of time bin on WAKE on treatment day 8 ($F_{6, 108} = 13.5$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 108} = 9.3$, $p = 0.001$). Further analysis revealed that during the time bin 7pm-10pm, which coincided with the onset of the dark period, ethanol consumption decreased wakefulness($F_{2, 20} = 55.12$, $p < 0.001$) in comparison to control animals. No significant differences between the groups were found ($F_{2, 18} = 1.61$, $p = 0.23$).

2.4.5 Day 8 NREM:

As seen in **Figure 2.6b**, repeated ethanol withdrawal appeared to increased NREM sleep at 11am-1pm during the light phase compared with SWD rats and controls. Furthermore, ethanol consumption appeared to increase NREM at 7pm-10pm during the dark phase, compared with control rats, indicating that control rats were more active during the start of the dark phase.

A repeated measures ANOVA revealed a significant main effect of time bin on NREM on treatment day 8 ($F_{6, 96} = 15.5$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 96} = 10.7$, $p < 0.001$) consistent with the flattening of the curves for SWD and RWD groups in **figure 2.6B**. However, no significant group differences were found ($F_{2, 16} = 0.143$, $p = 0.87$) indicating that the total NREM over the day was unaffected.

2.4.6 Day 8 REM:

As demonstrated by **Figure 2.6c**, there did not appear to be a significant circadian rhythm in the treatment groups on day 8 of chronic ethanol treatment. A repeated measures ANOVA found no significant main effect of time bin on REM was found ($F_{6, 108} = 0.92$, $p = 0.38$), indicating that measures of REM were not affected across the day. No significant group x time bin interaction was found ($F_{12, 108} = 1.56$, $p = 0.23$) which indicates that the treatment groups did not behave differently as a function of time. No significant group differences were found in REM sleep ($F_{2, 18} = 1.07$, $p = 0.36$).

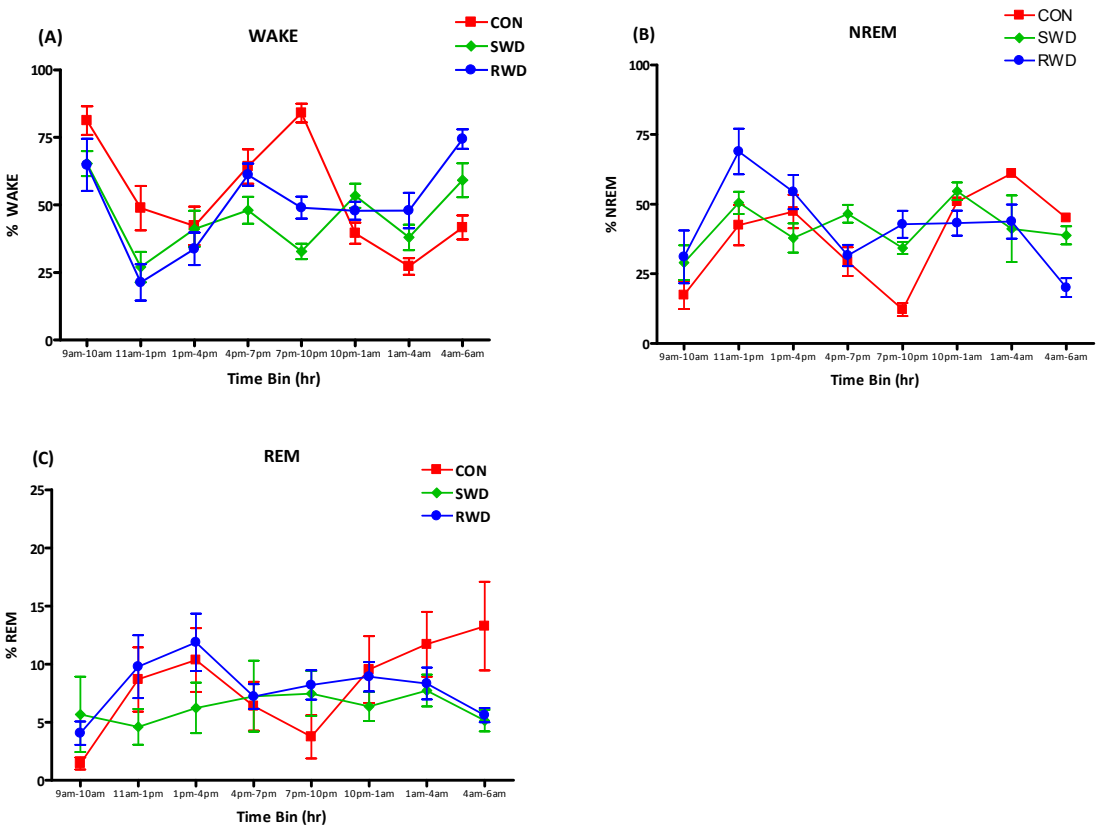


Figure 2.6: Mean measures of (A)% wake, (B) % NREM and (C) % REM sleep as measured by EEG / EMG recordings as taken on day 8 of chronic ethanol diet. Neither SWD or RWD rats had yet experienced a withdrawal episode.

2.4.7. Day 11 WAKE:

As depicted by **Figure 2.7a**, control rats showed a clear circadian rhythm of wakefulness, in which wakefulness increased from 11am to peak at the start of the dark phase and then decreased from 7pm to the start of the light phase. This showed a reversal in the normal rodent circadian rhythm, in which rats are expected to show high levels of wakefulness during the dark phase and low levels of wakefulness during the light phase.

Statistical analysis revealed a significant main effect of time bin on WAKE on treatment day 11 ($F_{6, 120} = 16.9$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 120} = 8.97$, $p = 0.001$). No significant overall differences between the groups were found ($F_{2, 20} = 0.36$, $p = 0.7$). Planned comparisons revealed that at 11am-1pm (t (20) = -9.5, $p < 0.001$), 7pm-10pm (t (20) = -7.7, $p < 0.001$) and 4am – 6am (t (20) = 4.7, $p < 0.001$), ethanol withdrawal (SWD and RWD groups) significantly decreased wakefulness. However, there were no further effects of

repeated ethanol withdrawal on wakefulness, at any of these specified time points 11am – 1pm (t (20) = -0.19, p = 0.85), 7pm-10pm (t (20) = -1.2, p = 0.23), 4am – 6am (t (20) = 0.49, p = 0.69). Hence, the first episode of withdrawal did not significantly alter wakefulness compared to the same exposure to chronic ethanol consumption.

2.4.8. Day 11 NREM:

As seen in **Figure 2.7b**, NREM was increased during the light phase in comparison to control rats, however, during the dark phase (1am – 6am), ethanol-fed rats show decreased NREM compared to controls. It appeared that chronic ethanol consumption leads to disruptions in the normal rodent circadian rhythm.

Statistical analysis using a repeated measures ANOVA revealed a significant main effect of time bin on NREM on treatment day 11 ($F_{6, 120}$ = 18.7, p < 0.001). A significant group x time bin interaction was found ($F_{12, 120}$ = 8.45, p < 0.001). Planned comparisons revealed that at 11am-1pm (t (20) = 3.0, p = 0.007), 4pm-7pm (t (20) = 4.7, p < 0.001), 1am – 4am (t (20) = -3.5, p = 0.002) and 4am-6am (t (20) = -2.8, p = 0.011) which indicated there was a significant effect of chronic ethanol consumption on NREM sleep at these time points. However, further analysis revealed no significant effects of the first ethanol withdrawal episode at these specified time points 11am-1pm (t (20) = 1.3, p = 0.22), 4pm-7pm (t (20) = 0.96, p = 0.35), 1am – 4am (t (20) = 0.81, p = 0.43) and 4am-6am (t (20) = -0.29, p = 0.77) indicating that despite there being a main effect of chronic ethanol consumption, the first episode of ethanol withdrawal did not significantly alter NREM sleep,

2.4.9 Day 11 REM:

As demonstrated by **Figure 2.7c**, REM sleep in the control group was increased from 9am – 1pm and subsequently decreased from 1pm to the start of the dark phase. There was a gradual increase in REM sleep from 7pm throughout the dark phase, indicating that the control rats had undergone 2 REM sleep peaks, one during the light phase (9am-7pm) and one in the dark phase (7pm-6am). Although there did not appear to be a significant difference between the treatment groups, the rats that were fed chronic ethanol diet showed disruption of circadian rhythm of REM sleep.

Statistical analysis using a repeated measures ANOVA found a significant main effect of time bin on REM was found on treatment day 11 ($F_{6, 120}$ = 4.5, p = 0.003, ε = 0.615), indicating that measures of REM were altered across the day. A significant group x time bin interaction was found ($F_{12, 120}$ = 4.87, p < 0.001, ε = 615) which indicates that the treatment groups behaved

differently as a function of time. However, no significant overall group differences were found ($F_{2, 20}$ = 2.63, p = 0.097, ε = 615).

Planned comparisons revealed that at the lights on and lights off time points, namely 4pm-7pm (t (20) = 3.3, p = 0.003) and 4am-6am (t (20) = -3.0, p = 0.007) a significant effect of chronic ethanol consumption on REM sleep was observed. Further analysis revealed a significant difference between the SWD and RWD groups at the lights off time point 4pm-7pm (t (20) = -2.0, p = 0.05) indicating the RWD group showed higher levels of REM compared to the SWD group at the onset of the dark phase. This finding suggests that at the onset of the dark phase, the first withdrawal episode increased REM sleep to higher levels than chronic ethanol consumption alone. However at the lights on time point, 4am-6am (t (20) = -0.98, p = 0.335) no linear trend was observed as the SWD group showed the lower levels of REM sleep compared to RWD group.

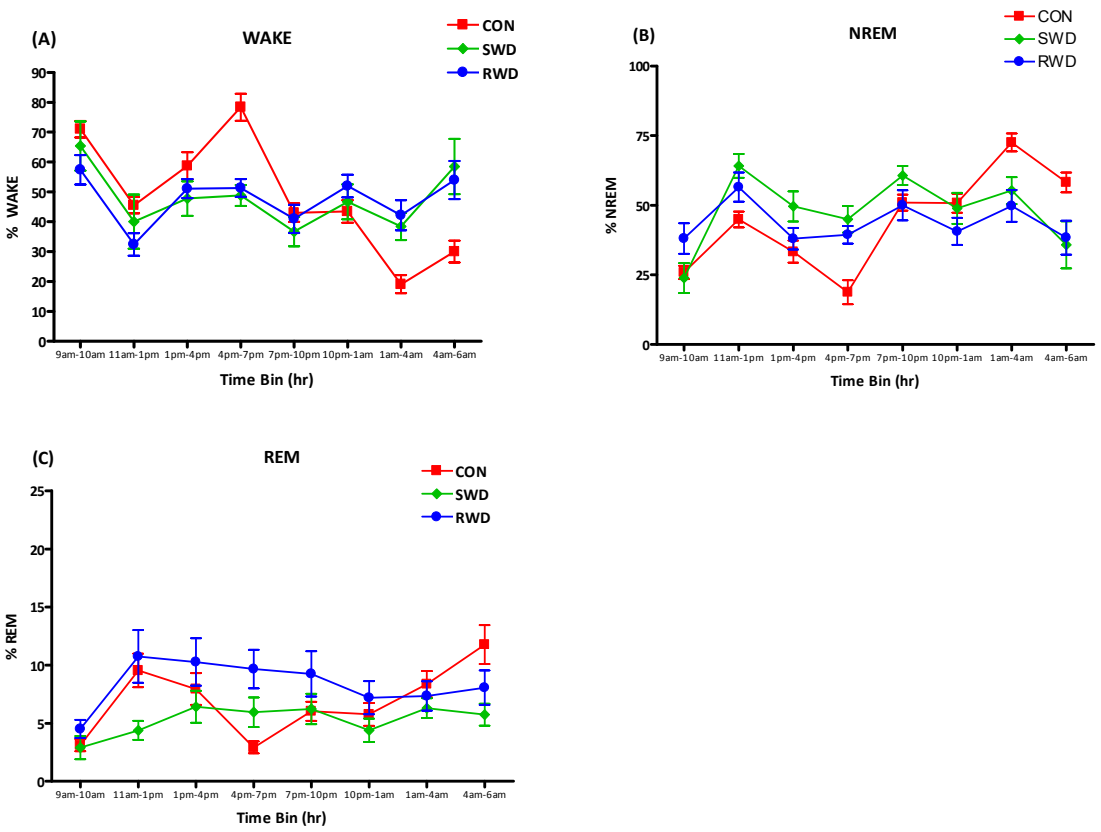


Figure 2.7: Mean measures of (A)% wake, (B) % NREM and (C) % REM sleep as measured by EEG / EMG recordings as taken on day 11 of chronic ethanol diet. For RWD group, Day 11 represented the first day of ethanol liquid diet after the first experience of an intermediate withdrawal episode.

2.4.10 Day 18 WAKE:

As depicted by **Figure 2.8a**, wakefulness levels in the control group was increased from 11am to the onset of the dark phase and subsequently decreased from 7pm to the onset of the light phase, after which wakefulness was moderately increased from 4am-6am, just prior to the lights on time point. It must be noted that this wakefulness pattern was not typical of the normal rat circadian rhythm as rats are generally more active during the dark phase. However, the typical wake pattern was not observed on treatment day 18. There was a "flattening out" of the wake pattern in the ethanol-fed rats which did not appear to follow the albeit altered circadian rhythm of the control rats.

A repeated measures ANOVA revealed a significant main effect of time bin on WAKE on treatment day 18 ($F_{6, 102} = 9.93$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 102} = 4.13$, $p < 0.001$).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant effect of chronic ethanol treatment at time bins 7pm-10pm (t (20) = 3.2, $p = 0.005$) and 1am-4am (t (17) = 3.9, $p = 0.001$). Further analysis comparing SWD vs. RWD groups show that at 7pm-10pm (t (20) = 2.5, $p = 0.023$) and 1am – 4am (t (17) = 2.5, $p = 0.021$) SWD rats showed significantly higher wakefulness than RWD rats. This result indicates that after the second withdrawal episode, although there is a significant effect of chronic ethanol consumption, the effect of the 2nd withdrawal episode gives rise to more moderate effects on WAKE in comparison to chronic ethanol consumption as experienced by the SWD group on treatment day 18.

2.4.11. Day 18 NREM:

As seen in **Figure 2.8b**, NREM sleep in all the treatment groups was increased from 11am to the onset of the dark phase, and there did not appear to be a difference between the treatment groups during the light phase. The control rats showed increased NREM sleep from 7pm to the onset of the light phase, however SWD and RWD rats showed decreased levels on NREM sleep compared to control rats, with RWD rats showing moderately elevated NREM levels compared to SWD rats. Similar to the wake patterns on day 18, the typical wake pattern was found to be disrupted in the CON rats, which exhibited higher levels of NREM during the dark phase compared to the light phase.

A repeated measures ANOVA revealed a significant main effect of time bin on NREM on treatment day 18 ($F_{6, 108} = 14.1$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 108} = 6.3$, $p < 0.001$).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 7pm-10pm (t (17) = -3.3, p = 0.004), 10pm-1am (t (17) = -2.1, p = 0.05) and 1am – 4am (t (17) = -3.2, p = 0.005) indicating that at these time points chronic ethanol consumption significantly decreased NREM sleep compared to controls. Further analysis comparing SWD vs. RWD groups at 7pm-10pm (t (17) = 2.5, p = 0.021) and 1am – 4am (t (17) = 2.2, p = 0.043) showed that SWD group showed significantly lower NREM sleep compared to RWD group. However at 10pm-1am (t (17) = 0.356, p = 0.73), there was no difference in NREM sleep between SWD and RWD groups.

2.4.12. Day 18 REM:

As demonstrated by **Figure 2.8c**, REM sleep in all treatment groups was increased from 11am throughout the course of the day, and there appeared to be no difference in REM sleep between the treatment groups.

Statistical analysis using a two way ANOVA revealed a significant main effect of time bin on REM was found on treatment day 18 ($F_{6, 96}$ = 4.4, p = 0.003), indicating that measures of REM were altered across the day, although the main effect of time bin did not interact significantly with the treatment group ($F_{12, 96}$ = 1.8, p = 0.086).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 4am- 6am (t (16) = -2.2, p = 0.045) indicating that chronic ethanol consumption significantly decreased REM sleep compared to controls at the onset of the light phase. Further analysis comparing SWD vs. RWD groups at 4am – 6am (t (16) = -0.33, p = 0.75) which revealed no significant differences in REM sleep between the SWD and RWD group.

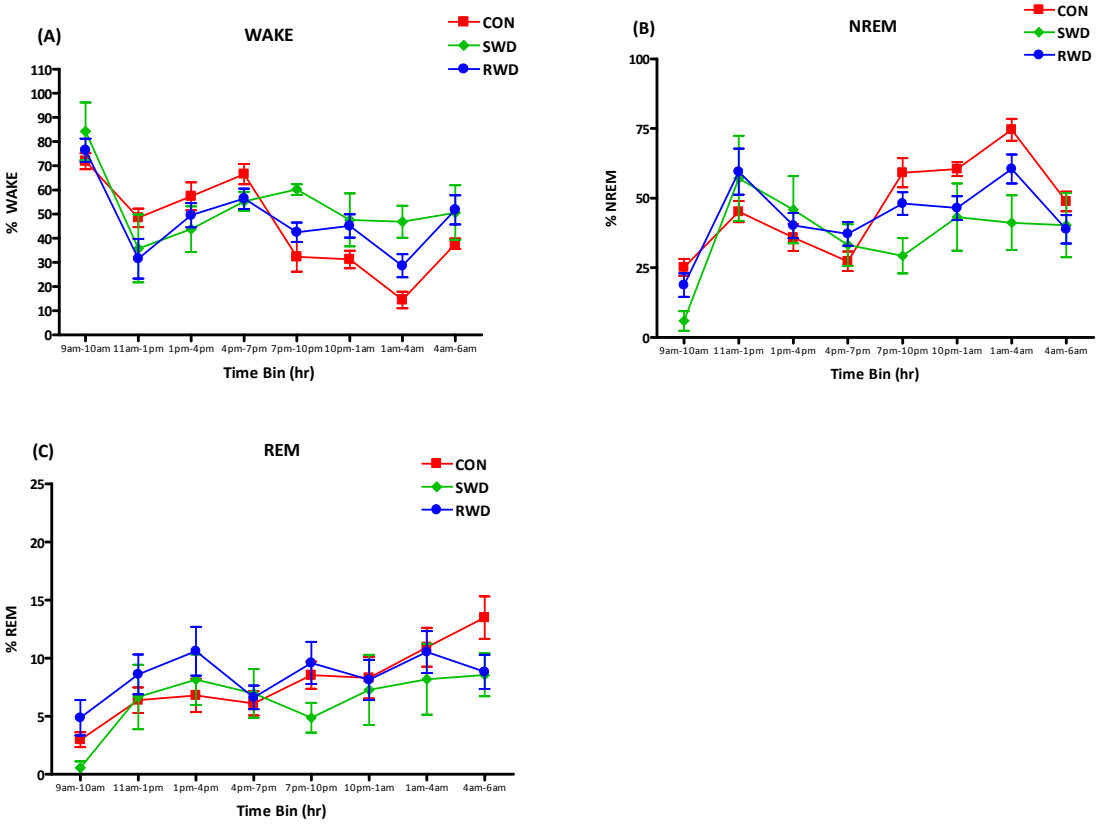


Figure 2.8: Mean measures of (A)% wake, (B) % NREM and (C) % REM sleep as measured by EEG / EMG recordings as taken on day 18 of chronic ethanol diet. For RWD group, Day 18 represented the first day of ethanol liquid diet after the 2nd intermediate withdrawal episode.

2.4.13. Day 24 (Final Withdrawal) WAKE:

As depicted by **Figure 2.9a**, on the final withdrawal day, CON rats show a peak in WAKE 1pm-4pm. Wakefulness decreased from 4pm to the onset of the light phase in CON animals. SWD and RWD rats appeared to follow similar circadian rhythms throughout the day. Both treatment groups showed a peak in WAKE at 4pm-7pm, but at lower WAKE levels compared to CON rats. During the dark phase, both SWD and RWD groups show increased wakefulness in comparison with CON rats. CON rats showed disrupted circadian patterns of wakefulness.

A repeated measures ANOVA found a significant main effect of time bin on WAKE on treatment day 24 ($F_{6, 96} = 7.5$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 96} = 8.8$, $p < 0.001$).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 11am- 1pm (t (16) = -2.5, $p = 0.025$), 1pm-4pm (t (16) = -5.1, $p = 0.04$), 10pm-1am (t (16) = 3.7, $p = 0.002$), 1am-4am (t (16) = 3.6, $p = 0.002$), 4am-6am (t (16) = 3.3, $p = 0.004$)

indicating that chronic ethanol consumption significantly altered wake patterns compared to controls at these specified time points. Further analysis comparing SWD vs. RWD groups revealed that at 10pm-1am (t (16) = 4.01, p = 0.001) the SWD group showed significantly increased wakefulness compared to the RWD group. There were no significant differences in wakefulness between SWD and RWD groups at 11am-1pm (t (16) = -0.025, p = 0.98), 1pm-4pm (t (16) = 0.084, p = 0.93), 1am-4am (t (16) = 0.65, p = 0.53) and 4am-6am (t (16) = 0.092, p = 0.93).

2.4.14. Day 24 (Final Withdrawal) NREM:

As seen in **Figure 2.9b**, in control rats, NREM sleep decreased from 11am-4pm and subsequently increased and peaked at 1am-4am, during the dark phase after which REM sleep moderately decreased at the onset of the light phase. During the light phase, both SWD and RWD rats showed a decreased NREM during the light phase and exhibited lower levels of NREM sleep during the dark phase compared to CON rats. Control rats did not demonstrate the typical sleep/wake patterns and showed disrupted NREM sleep patterns.

A repeated measures ANOVA found a significant main effect of time bin on NREM on treatment day 24 ($F_{6, 102}$ = 8.15, p < 0.001). A significant group x time bin interaction was found ($F_{12, 102}$ = 11.3, p < 0.001). However, no significant group differences were found in NREM sleep ($F_{2, 17}$ = 0.16, p = 0.86).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 11am- 1pm (t (16) = -2.5, p = 0.025), 1pm-4pm (t (16) = -5.1, p = 0.04), 10pm-1am (t (16) = 3.7, p = 0.002), 1am-4am (t (16) = 3.6, p = 0.002), 4am-6am (t (16) = 3.3, p = 0.004) indicating that chronic ethanol consumption significantly altered NREM patterns compared to controls at these specified time points. Further analysis comparing SWD vs. RWD groups revealed that at 10pm-1am (t (16) = 4.01, p = 0.001) the SWD group showed significantly increased NREM compared to the RWD group. There were no significant differences in NREM between SWD and RWD groups at 11am-1pm (t (16) = -0.025, p = 0.98), 1pm-4pm (t (16) = 0.084, p = 0.93), 1am-4am (t (16) = 0.65, p = 0.53) and 4am-6am (t (16) = 0.092, p = 0.93).

2.4.15. Day 24 (Final Withdrawal) REM:

As demonstrated by **Figure 2.9c**, CON rats show a general increase in REM sleep throughout the day, with the peak REM sleep occurring during the dark phase. SWD and RWD rats also show a general increase in REM sleep across the course of the day but to a lesser extent compared with CON during the dark phase.

A repeated measures ANOVA revealed a significant main effect of time bin on REM was found on treatment day 24 ($F_{6, 96} = 11.2$, $p < 0.001$), indicating that measures of REM were altered across the day. A significant group x time bin interaction was found ($F_{12, 96} = 3.7$, $p < 0.001$).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 1am-4am (t (16) = -2.8, p = 0.013) and 4am-6am (t (16) = -2.4, p = 0.03) indicating that chronic ethanol consumption significantly altered REM sleep compared to controls at these specified time points. Further analysis comparing SWD vs. RWD groups revealed that at 1am-4am (t (16) = -0.67, p = 0.52) and 4am-6am (t (16) = -0.37, p = 0.72) there was no significant difference in REM sleep between the SWD and RWD groups.

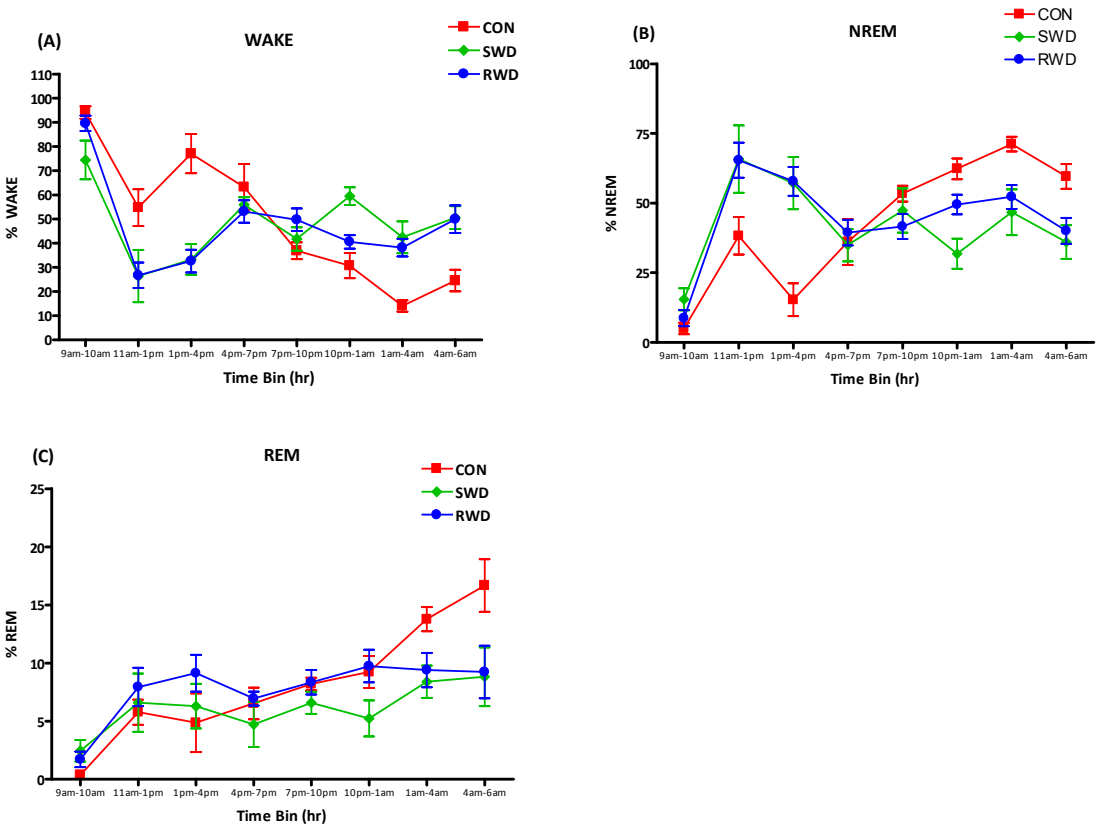


Figure 2.9: Mean measures of (A) % wake, (B) % NREM and (C) % REM sleep as measured by EEG / EMG recordings as taken on day 24 of chronic ethanol diet. All rats experienced the final withdrawal from liquid diet on day 24.

2.4.16. Post withdrawal day 3 WAKE:

As depicted by **Figure 2.10a**, control rats showed a peak in WAKE at 4pm – 7pm, the onset of the dark phase, followed by decreased WAKE during the dark phase, indicating that CON rats continued to demonstrate altered circadian rhythm. SWD and RWD groups showed a

"flattening out" of circadian rhythm, and showed higher WAKE levels at the onset of the light phase compared to CON rats.

Statistical analysis revealed a significant main effect of time bin on WAKE on treatment day 24 ($F_{6, 84} = 10.4$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 84} = 4.8$, $p < 0.001$).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 1am-4am (t (14) = 4.3, $p = 0.001$) and 4am-6am (t (14) = 4.2, $p = 0.001$) indicating that chronic ethanol consumption significantly altered wakefulness compared to controls at these specified time points. Further analysis comparing SWD vs. RWD groups revealed that at 1am-4am (t (14) = 2.9, $p = 0.013$) SWD rats showed significantly higher level of wakefulness compared to RWD rats whereas 4am-6am (t (14) = 1.7, $p = 0.114$) there was no significant difference in wakefulness of the SWD and RWD groups.

2.4.17. Post withdrawal day 3 NREM:

As seen in **Figure 2.10b**, control rats showed a moderate increase in NREM sleep at 11am-4pm, followed by a decrease in NREM at, at the onset of the dark phase. During the dark phase, NREM sleep increased in CON rats until the onset of the light phase. SWD rats show a relatively stable level of NREM throughout the course of the day whereas RWD rats show a decrease in NREM at 11am to the onset of the dark phase. NREM peaked in RWD rats at 1am-4am. Both SWD and RWD groups show lower levels of NREM sleep compared to CON rats during the dark phase.

A repeated measures ANOVA revealed a significant main effect of time bin on NREM on treatment day 24 ($F_{6, 90} = 11.02$, $p < 0.001$). A significant group x time bin interaction was found ($F_{12, 90} = 5.7$, $p < 0.001$).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 1am-4am (t (15) = -3.3, $p = 0.005$) and 4am-6am (t (15) = -4.5, $p < 0.001$) indicating that chronic ethanol consumption significantly altered NREM compared to controls at these specified time points. Further analysis comparing SWD vs. RWD groups revealed that at 1am-4am (t (15) = -1.8, $p = 0.095$) and 4am-6am (t (15) = -1.4, $p = 0.19$) there was no significant difference in NREM of the SWD and RWD groups.

2.4.18. Post withdrawal day 3 REM:

As demonstrated by **Figure 2.10c**, during the light phase, CON rats show decreased REM sleep. CON rats showed an increase in REM sleep during the dark phase, peaking at 4am-6am. Both SWD and RWD groups showed a decrease in REM sleep from 11am to the onset of the dark phase, after which SWD rats demonstrated a "flattening out" of REM sleep at during the dark phase whereas RWD rats showed an increase in REM during the dark phase, after which NREM in RWD rats decreased marginally.

A repeated measures ANOVA found a significant main effect of time bin on REM sleep on treatment day 24 which was the final withdrawal day ($F_{6, 84} = 5.51$, $p = 0.004$), indicating that measures of REM were altered across the day. A significant group x time bin interaction was found ($F_{12, 84} = 3.35$, $p = 0.01$).

Planned comparisons comparing CON vs. SWD and RWD groups revealed a significant difference at 11am-1pm (t (14) = 2.3, $p = 0.04$) indicating that chronic ethanol consumption significantly altered REM compared to controls at these specified time points. Further analysis comparing SWD vs. RWD groups revealed that at 11am-1pm (t (14) = 0.74, $p = 0.47$) indicating that there was no significant difference in REM of the SWD and RWD groups.

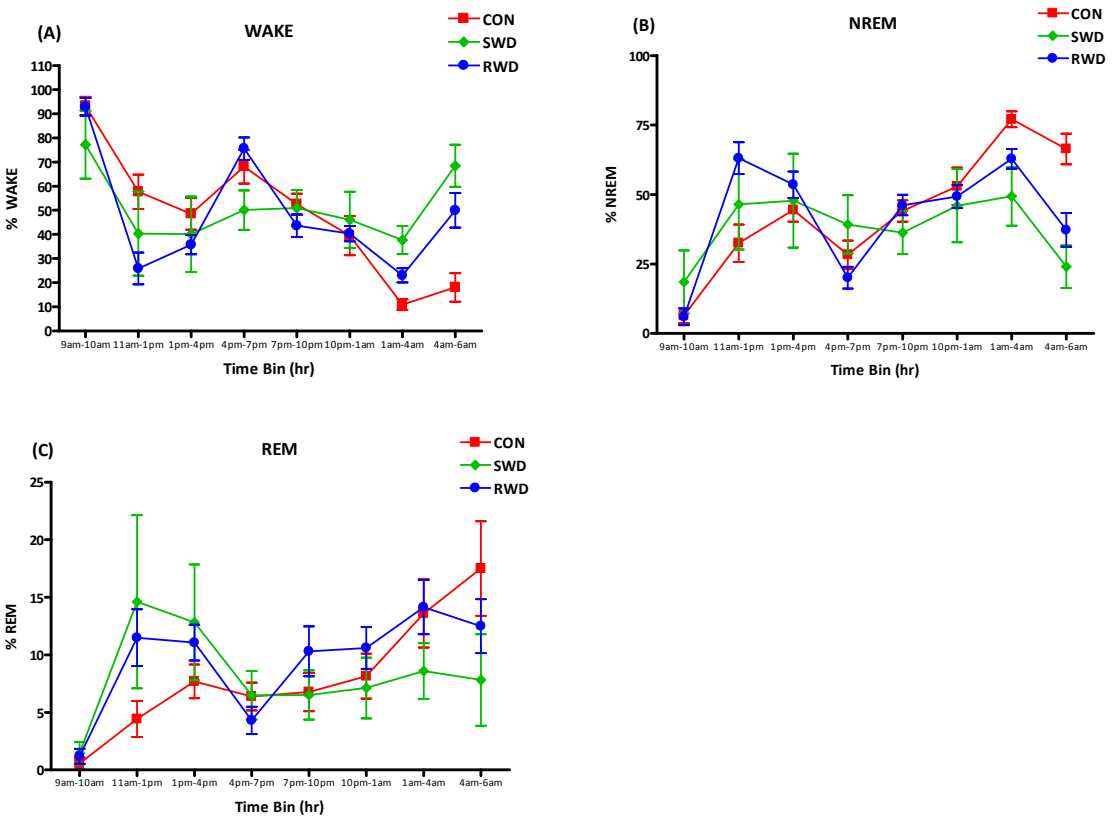


Figure 2.10: Mean measures of (A)% wake, (B) % NREM and (C) % REM sleep as measured by EEG / EMG recordings as taken on post-withdrawal day 3 of chronic ethanol diet. This time point represented the 3rd day after the final withdrawal.

2.5. Activity:

Figures 2.11 and 2.11 shows the mean activity levels for the duration of chronic ethanol treatment and in withdrawal. Repeated measures ANOVA indicated a significant main effect of treatment day ($F_{5, 100} = 12.8$, $p < 0.001$), and a group x treatment day interaction ($F_{10, 100} = 4.89$, $p < 0.001$). Further analysis found that compared with baseline measurements, overall activity levels on treatment day 8 ($p = 0.002$), day 11 ($p = 0.023$), day 18 ($p < 0.001$) and day 24 (final withdrawal day) ($p = 0.001$) were significantly attenuated. However, these effects appeared to dissipate 3 days after the final withdrawal when no significant differences were observed in activity levels compared with baseline ($p = 1.0$).

There was also a significant main effect of time bin ($F_{6, 120} = 25.8$, $p < 0.001$). Further analysis revealed that all rats showed the highest level of activity during 4pm-7pm, which coincided with the onset of the dark phase. There was a significant group x time bin interaction ($F_{12, 120} = 9.6$, $p < 0.001$). Further analysis revealed that although there was no significant main effect of treatment group ($F_{2, 20} = 0.11$, $p = 0.89$), at the time bin 4pm-7pm, there was a significant group difference in activity levels on day 11 (i.e. after the 1st intermediate withdrawal episode for the RWD group) ($F_{2, 20} = 6.8$, $p = 0.006$) in which post hoc tests showed that CON rats were significantly more active than SWD ($p = 0.024$) and RWD ($p = 0.009$) rats, however no significant difference between the ethanol-fed rats was observed ($p = 1.0$).

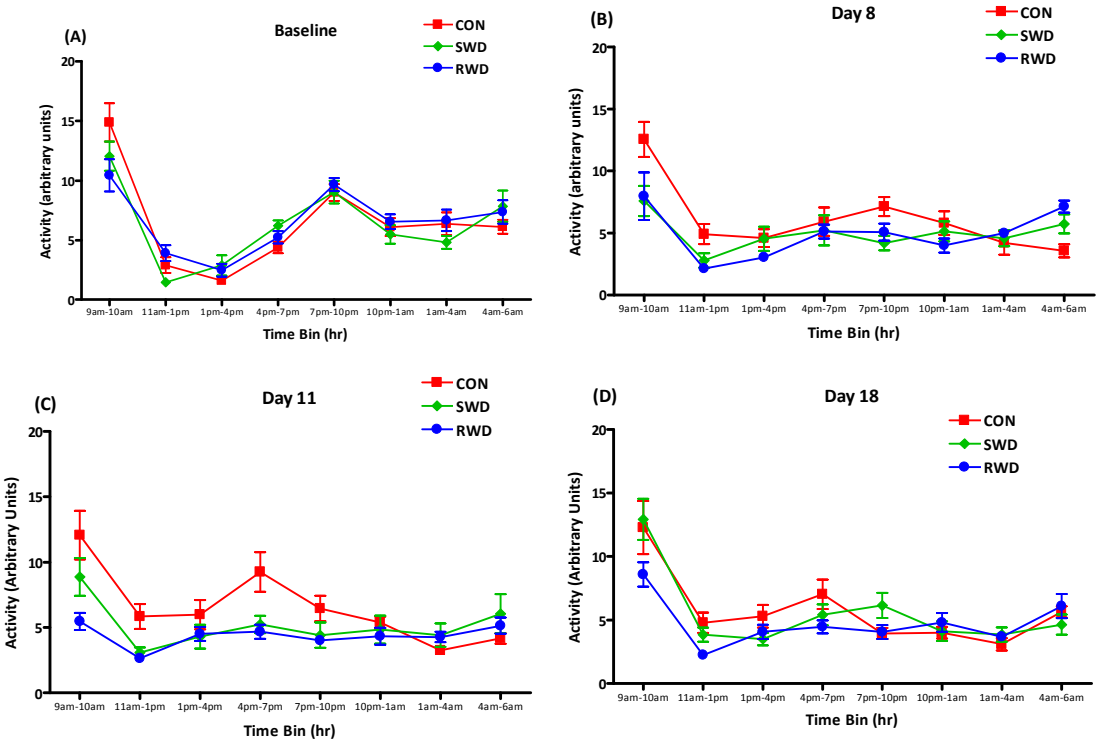


Figure 2.11: Mean measures of activity at (A) baseline (B) treatment day 8, (C) treatment day 11 (RWD experienced 1st withdrawal episode) and (D) treatment day 18 (RWD experienced 2nd withdrawal episode).

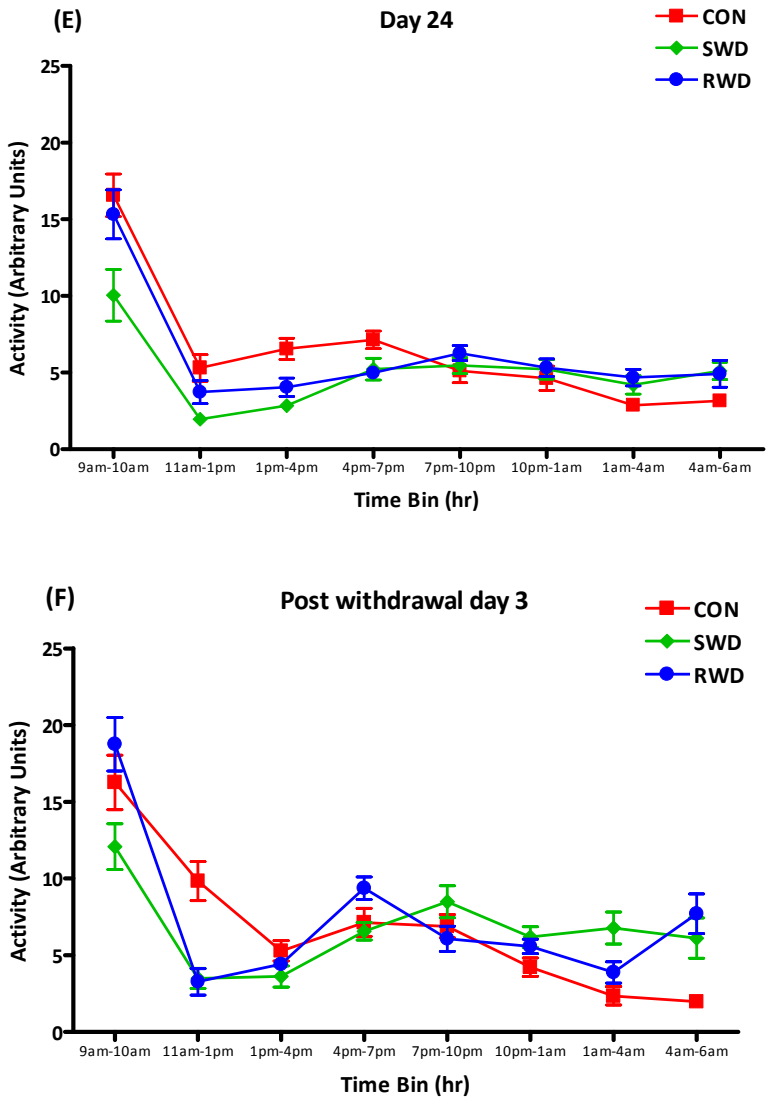


Figure 2.12: Mean measures of activity at (E) final withdrawal day (treatment day 24) and (F) Post withdrawal day 3.

2.6. Temperature:

Figures 2.13 and 2.14 shows the mean body temperatures for the duration of chronic ethanol treatment and in withdrawal. Repeated measures ANOVA did not find a significant main effect of treatment day ($F_{5, 100} = 2.26$, $p = 0.14$), and no significant group x treatment day interaction ($F_{10, 100} = 0.19$, $p = 0.86$).

There was however, a significant main effect of time bin ($F_{6, 120} = 32.1$, $p < 0.001$) which interacted significantly with treatment group ($F_{12, 120} = 7.75$, $p < 0.001$) suggesting that alcohol treatment produces body temperature changes at various times of the day. However posthoc analysis revealed no significant differences between the treatment groups ($F_{2, 20} = 0.02$, $p =$

0.98) indicating that rats in withdrawal do not experience significant changes in body temperature.

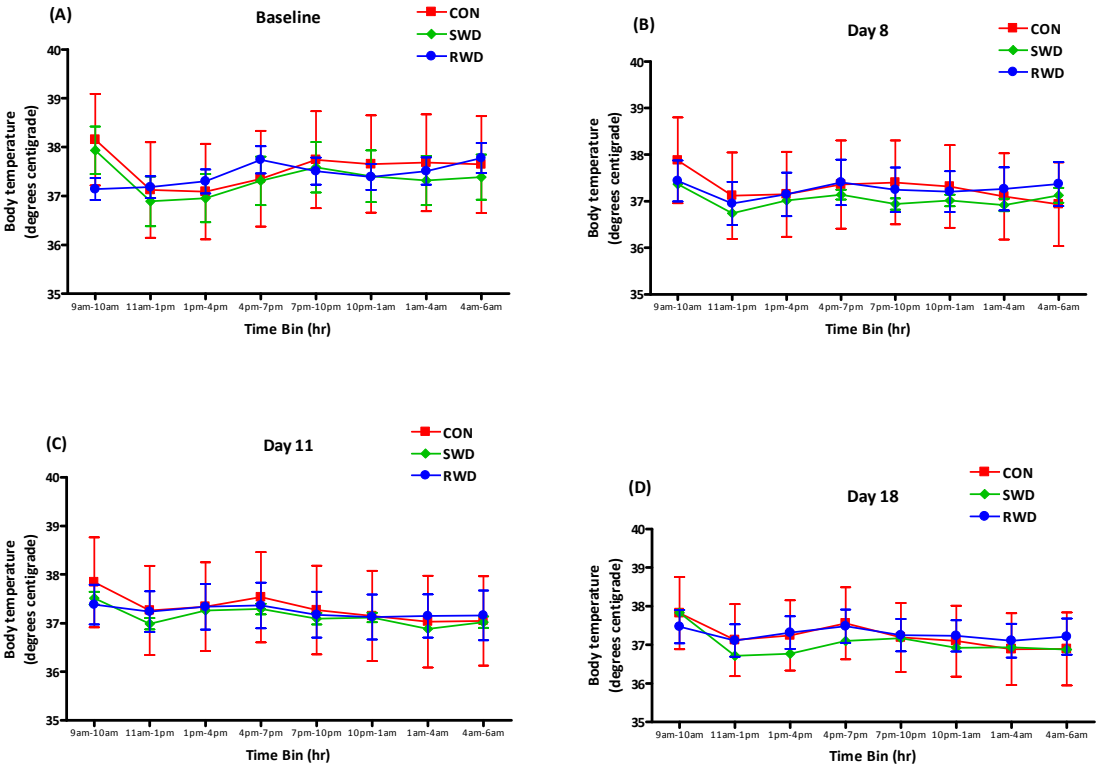


Figure 2.13: Mean measures of body temperature at (A) baseline (B) treatment day 8, (C) treatment day 11 (RWD experienced 1st withdrawal episode) and (D) treatment day 18 (RWD experienced 2nd withdrawal episode).

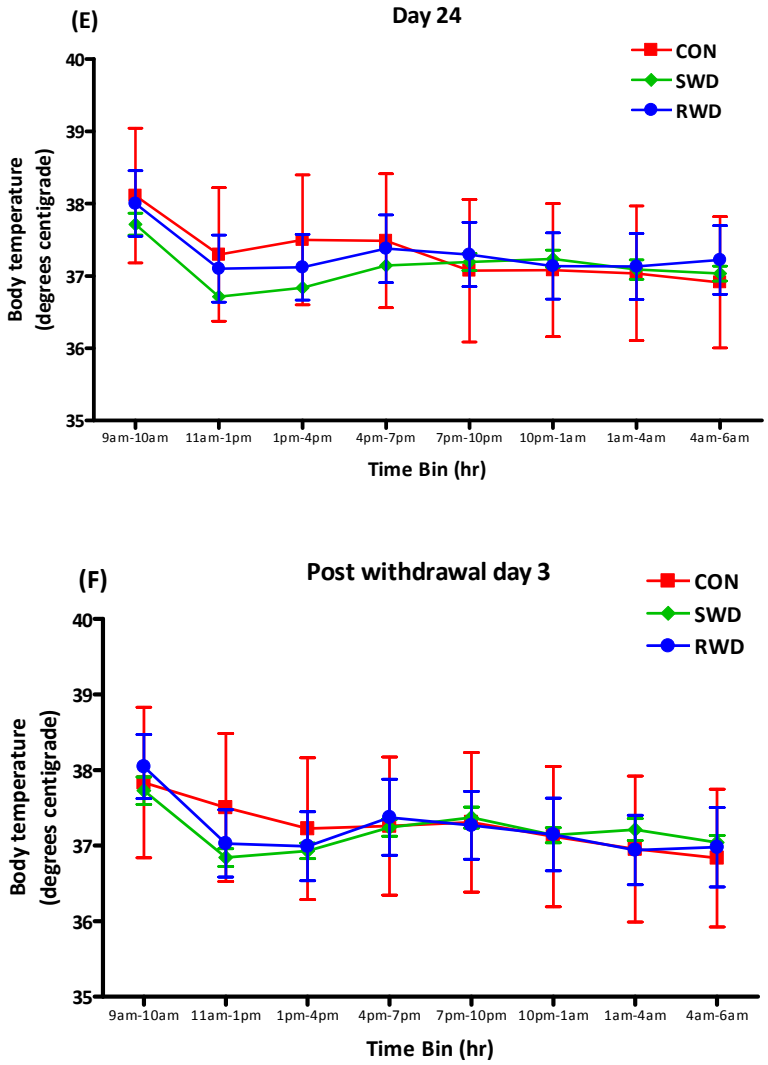


Figure 2.14: Mean measures of body temperature at (E) final withdrawal day (treatment day 24) and (F) Post withdrawal day 3.

2.7. Experiment 2: Post withdrawal food and water intake:

The mean ethanol consumption over the final 7 days of chronic ethanol treatment (following the last intermediate withdrawal episode in the RWD group) was 9.46 ± 0.18 g ethanol per kg body weight per day in the SWD group and 8.78 ± 0.14 g ethanol per kg body weight in the RWD group. A One Way ANOVA over this period revealed that there was no significant difference in ethanol intake across the two groups ($F_{1, 4} = 0.84$, $p = 0.41$). However, ethanol intakes in this experiment were markedly lower than on experiment 1 of this chapter.

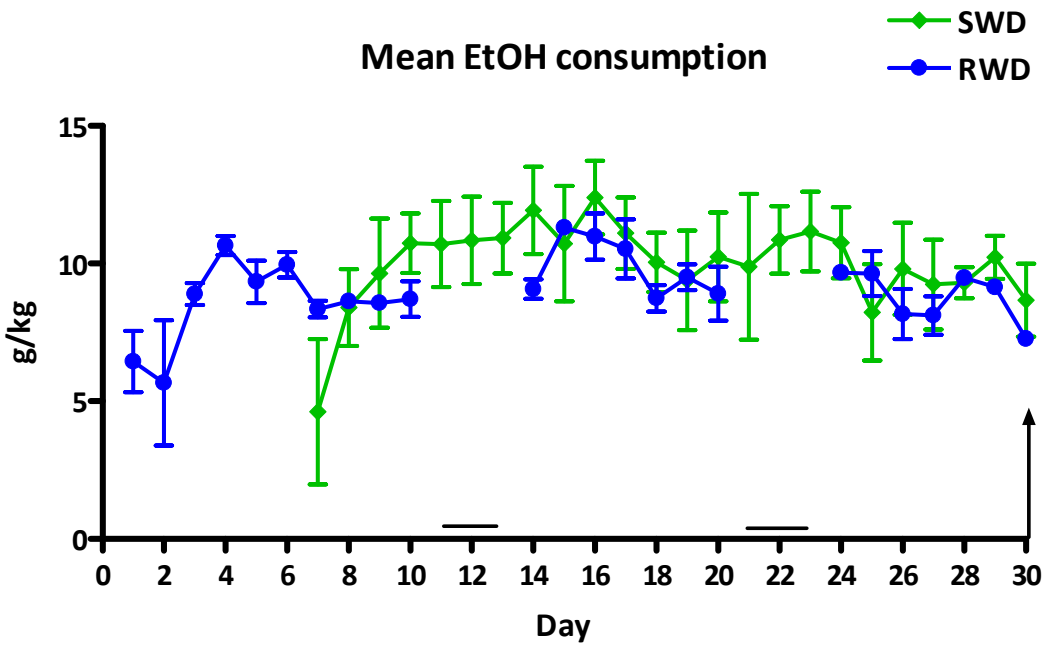


Figure 2.15: Mean ethanol consumption expressed as g per kg of body weight per experimental day. Arrow indicates the final withdrawal, intermediate withdrawal episodes for the RWD group occurred on experimental days 11-13 and 21-23. There was no overall difference in ethanol consumption between SWD and RWD groups.

As demonstrated by **Figure 2.15**, there was no significant difference in ethanol consumption between single and repeated withdrawal groups (p = 0.18). SWD and RWD groups consumed an average of 9 g/kg which is considerably lower than seen in previous studies conducted

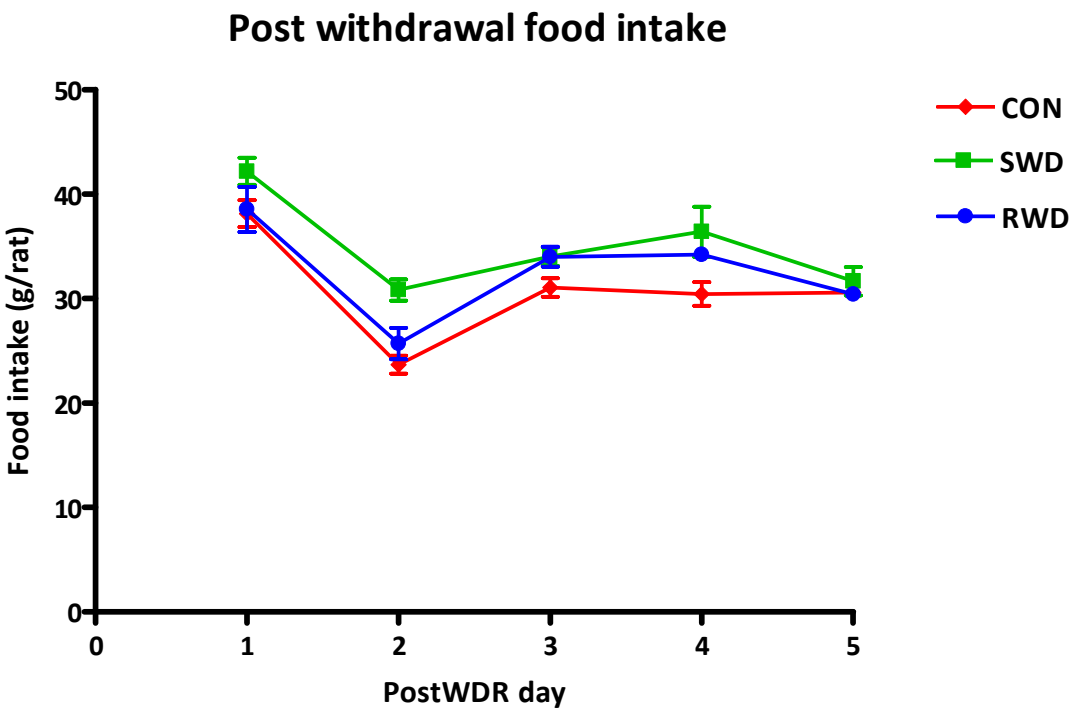


Fig 2.16: Post withdrawal food intake. Data expressed as Mean±SEM weight of rat chow consumed by CON group (red), SWD group (green) and RWD group (blue) measured for 5 days following the final withdrawal day.

As illustrated by **Fig 2.16**, the average post-withdrawal food intake on day 1 after the final withdrawal was 39.6 ± 0.95 g per rat. This decreased on the 2nd day after the final withdrawal and marginally increased on the 3rd post-withdrawal day after which remained at relatively stable consumption levels between 30 – 33 g per rat.

A repeated measures ANOVA found a significant main effect of day on post withdrawal food intake ($F_{4, 24}$ = 20.9, p <0.001). The main effect of treatment day did not interact significantly with group ($F_{8, 24}$ = 0.68, p =0.7). There was no significant group effect in post withdrawal food intake ($F_{2, 6}$ = 3.1, p = 0.12) indicating that RWD did not undergo greater withdrawal severity compared with SWD rats.

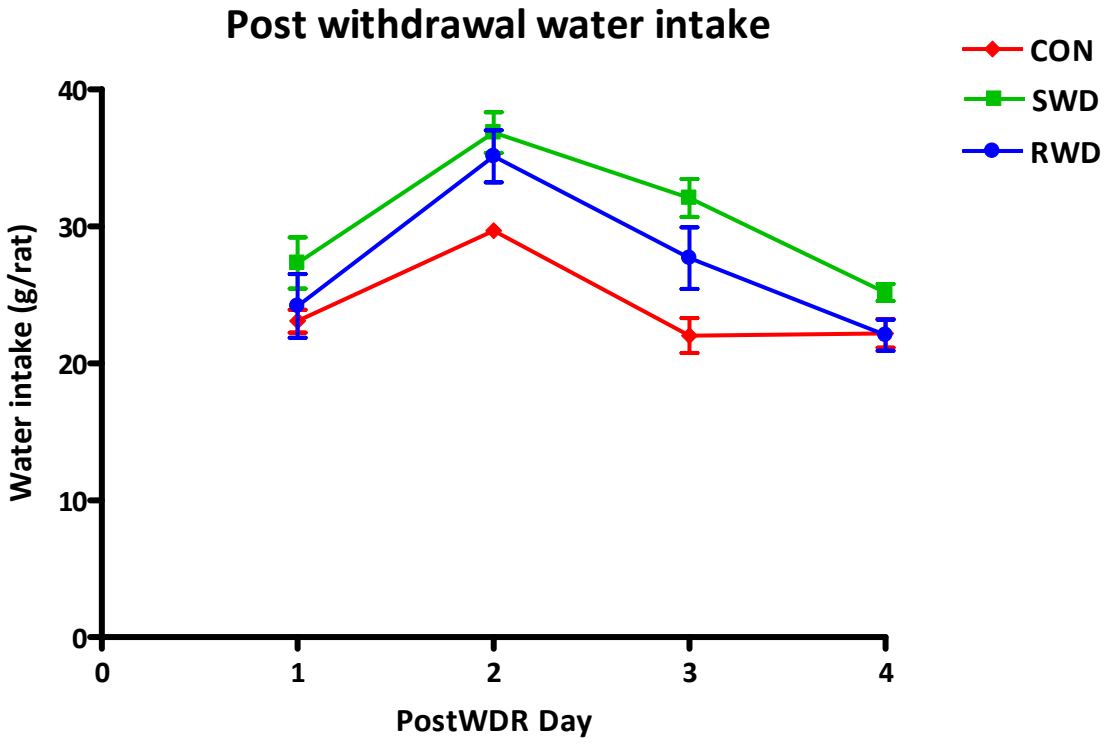


Fig 2.17: The effects of repeated ethanol withdrawal on post withdrawal water intake. Water intake was measured by the daily weighing of water bottles in a 24-hour period. Data expressed in Mean±SEM g of water drunk per rat.

Figure 2.17 shows the post-withdrawal water intakes of CON, SWD and RWD rats. On the 1st day after withdrawal, rats drank on average 24.9 ± 0.94 g of water. Water intake of all the rats increased on day 2, and SWD and RWD rats showed higher water intakes compared to CON rats on days 2 and 3. However, by post-withdrawal day 4, the water intake of the rats across the treatment groups had equalised to a mean of 23.1 ± 0.5 g of water.

A repeated measures ANOVA revealed a significant main effect of day on post withdrawal water intake ($F_{3, 45} = 82.2$, $p < 0.001$). There was a significant group x day interaction ($F_{6, 45} = 4.08$, $p = 0.002$). There was a significant group effect in post withdrawal water intake ($F_{2, 15} = 7.4$, $p = 0.006$). SWD group showed highest overall levels of post withdrawal water intake compared with CON group ($p = 0.005$) but there were no significant group differences in post withdrawal water consumption between SWD and RWD groups ($p = 0.21$). All the groups showed a decline in water intakes on post withdrawal day 3. However, the SWD group showed a smaller decline in water intakes compared with both RWD and CON groups.

2.8. Discussion:

2.8.1. Overview of Blood ethanol concentrations (BEC)

In this present investigation, there were no significant differences in the ethanol consumption between SWD and RWD groups. SWD group drank an average of 21.6 ± 0.63 g / kg / day of ethanol and RWD group drank an average of 21.3 ± 0.77 g / kg / day of ethanol (see **figure 2.2**) in the last 7 days of chronic ethanol treatment, which represented a high level of ethanol intake compared with previous studies conducted in this laboratory. Blood ethanol levels (see **figure 2.4**) were assessed to investigate the stability of blood ethanol levels attained by ethanol liquid diet administration and to assess the effects of withdrawal on blood ethanol concentrations. No significant differences in blood ethanol concentrations were found between SWD and RWD groups.

2.8.2. Overview of Post Withdrawal Food and Water Intake

Measurements of post-withdrawal food and water intake was used to assess withdrawal severity in rats (see **figure 2.16 and 2.17**); ethanol-fed rats drank more water during withdrawal, indicating an effect of ethanol withdrawal, which lasted for 4 days compared with CON rats. However, this did not provide evidence that repeated episodes of ethanol withdrawal led to greater severity of withdrawal symptoms as a single withdrawal from ethanol resulted in higher water intakes than repeated ethanol withdrawal. Measurements of core body temperature (see **figure 2.13 and 2.14**) and activity (see figure **2.11 and 2.12**) as recorded using telemetric methods reveal no significant group differences.

2.8.3. Overview of EEG / EMG Measurements of Sleep states

There were no significant group differences in WAKE, NREM and REM sleep at baseline (see **figure 2.5**). By treatment day 8, chronic ethanol consumption appeared to result in a moderate "flattening out" of circadian rhythm, particularly of WAKE and NREM sleep, across the course of the day which was also observed on day 11 (during which RWD group had experienced their 1st intermediate withdrawal episode), day 18 (during which RWD group had experienced their 2nd intermediate withdrawal episode), day 24 (represents the final withdrawal day on which all rats were withdrawn from liquid diet and fed rat chow) and post withdrawal day 3 (represents the 3rd day after the final withdrawal). There was a significant main effect of chronic ethanol consumption on sleep / wake measures in both SWD and RWD rats on day 11. The first withdrawal episode in RWD rats had no effect on wakefulness or NREM sleep. However, REM

sleep in RWD group was found to be significantly increased during the dark phase compared with SWD group.

By day 18, when RWD group had experienced a 2nd intermediate withdrawal, there was found to be no significant effect of repeated ethanol withdrawal on WAKE and REM sleep, although SWD rats showed decreased NREM sleep during the dark phase compared to both CON and RWD rats. However, by treatment day 18, alterations in the circadian rhythm of the CON rats had started to emerge, demonstrating low levels of wakefulness during the dark phase and high levels of NREM during the dark phase.

By day 24, the final withdrawal day, although there was a main effect of chronic ethanol consumption, there was no significant effect of RWD on wakefulness, NREM and REM sleep. By post-withdrawal day 3, SWD rats showed significantly higher levels of wakefulness during the dark phase compared to CON and RWD rats, however, no significant differences in NREM and REM emerged. These findings taken together indicate that chronic ethanol consumption led to disruption of the normal rat circadian rhythm during the course of the day, however any differences in sleep / wake measures as a result of repeated ethanol withdrawal did not appear to be sufficiently robust to make firm conclusions regarding greater withdrawal severity.

Sleep architecture and circadian rhythm were disrupted (see **figure 2.5 – 2.10**) as a result of chronic ethanol consumption, especially in measures of wakefulness and NREM sleep but this effect did not appear to be exacerbated by multiple episodes of withdrawal. However, data analysis and interpretation was complicated by the finding that administration of control liquid diet to the control rats altered the normal circadian rhythm and hence had implications in data interpretation.

2.8.4. Main Discussion

The present study set out to examine the effects of repeated ethanol withdrawal on several behavioural measures of ethanol withdrawal severity. Using an automated computerised radio-telemetric system to determine measures of EEG / EMG, core body temperature and activity levels simultaneously, allowed continuous monitoring of physical signs of ethanol withdrawal. Additionally, blood ethanol concentrations were measured during the course of chronic ethanol treatment and food and water intake were measured during the post-withdrawal period to assess any withdrawal-related effects. The results from the present study did not suggest that repeated ethanol withdrawal led to greater severity of withdrawal

symptoms in comparison to single ethanol withdrawal. These findings were unexpected as repeated ethanol withdrawal is consistently associated with increased withdrawal severity on subsequent withdrawals in human alcoholics (Ballenger and Post 1978; Baker and Cannon 1979; Brown, Anton et al. 1988) and animal models of ethanol dependence and withdrawal (Becker and Hale 1993; Becker 1994; Becker, Diaz-Granados et al. 1997; Becker, Diaz-Granados et al. 1997). However, it is important to note that these previous studies focused on the measurement seizure thresholds, which serves as an indicator for possible increased withdrawal severity rather than home-cage behaviour and sleep architecture as measured in the current experiments. Thus, the findings of this current thesis indicate that measures of withdrawal severity using seizure sensitivity may not generalise to other behavioural measures of withdrawal.

Although in this current study, repeated ethanol withdrawal did not result in greater withdrawal severity, our rat model of repeated ethanol withdrawal has replicated similar withdrawal-related behaviours observed in human alcoholics. Stephens et al (2001) reported that rats with previous withdrawal experience showed deficits in learning associations between neutral stimuli and aversive events. In human alcoholics who have experienced repeated detoxifications, impairments in fear conditioning and fear conditioning generalisation to related stimuli have been observed (Stephens, Ripley et al. 2005). This evidence suggests altered function of the amygdala, which is consistent with findings that alcoholic in-patients with experience of more than 2 medically supervised detoxifications experience more interference from words associated with emotional experiences with alcohol (Duka, Townshend et al. 2002). Repeated ethanol withdrawal appears to be associated with learning deficits, including negative patterning discrimination (Borlikova, Elbers et al. 2006) which is thought to involve configural learning, interfered with the acquisition of lever pressing on a V160 schedule (Stephens, Brown et al. 2001).

Although in the current investigation, there were no significant differences in ethanol intake between the SWD and RWD groups, it should be noted that in the second experiment (i.e. measurement of post withdrawal food and water intake) lower ethanol consumption observed in the ethanol-fed rats than ethanol consumption levels attained in previous studies conducted in this laboratory was an important limitation.

In this current study, there was little evidence to support the hypothesis that repeated ethanol withdrawal led to more profound sleep disruptions although there was evidence of disrupted sleep patterns following chronic ethanol consumption on EEG / EMG measures. These current

findings suggest that chronic ethanol consumption leads to disruption of the normal rat circadian rhythm across the course of the day, however any differences in sleep / wake measures between SWD and RWD rats as a result of repeated ethanol withdrawal did not produce a sufficiently robust effect to allow firm conclusions to be made regarding greater severity of ethanol withdrawal.

Despite this current finding, clinical studies have found alcoholics with a history of previous alcohol detoxifications were more likely to experience insomnia and other sleep difficulties related to the withdrawal experience (Brown, Anton et al. 1988; Gillin, Smith et al. 1990; Moak and Anton 1996; Worner 1996). These clinical findings have been supported by several animal studies, reporting reduced sleep time during ethanol withdrawal after chronic ethanol exposure (Mendelson, Majchrowicz et al. 1978; Rouhani, Emmanouilidis et al. 1990; Rouhani, Dall'Ava-Santucci et al. 1998; Ehlers and Slawecki 2000; Kubota, De et al. 2002). Although the effects of ethanol on sleep architecture in animal models are more difficult to determine and findings in animal models have been less consistent. Mendelson and colleagues have reported increases in REM sleep (Mendelson, Majchrowicz et al. 1978), whereas Rouhani et al have found decreased REM as a result of chronic ethanol consumption (Rouhani, Emmanouilidis et al. 1990). Kubota and associates found rats undergo circadian variation during ethanol withdrawal (Kubota, De et al. 2002) which may explain the disruptions in sleep/ wake cycles in this present study associated with ethanol withdrawal compared with control rats. However, the inconsistencies between these studies may be attributed to the procedural differences among these experiments such as the method and duration of ethanol administration, the blood ethanol concentrations attained and the duration of data collection. Furthermore, these studies did not specifically investigate the effects of repeated episodes of ethanol withdrawal. In a mouse study by Veatch which investigated the sleep time and sleep architecture in a mouse model of repeated chronic ethanol exposure and withdrawal, it was found that withdrawal after chronic ethanol exposure led to profound disruptions in sleep composition with a reduction in NREM sleep and increased REM sleep (Veatch 2006), which was consistent with the “REM rebound” phenomenon which involves an increased pressure for REM sleep due to prior deprivation of REM sleep (Williams and Rundell 1981; Drummond, Gillin et al. 1998). The findings reported by Veatch (Veatch 2006) were partially replicated in this current experiment as repeated ethanol withdrawal led to an overall decrease in NREM sleep however, it must be noted that the increased REM sleep as observed in Veatch’s study was not found in this present experiment.

One possible explanation for this difference could have been associated with the experimental differences in ethanol administration. In Veatch's experiment, mice were exposed for four cycles of ethanol vapour lasting 16-hours, separated by 8-hour periods of withdrawal, and mice attained a blood ethanol concentration of 165 mg%. In this present experiment, rats were fed 7% ethanol containing liquid diet for a total of 30 days, a period which was interspersed with 2 intermediate withdrawal episodes lasting for 3 days each. On average, blood ethanol concentrations attained were 8.4 ± 2.09 mmol / L (38.7 mg / dL) for the SWD group and 10.4 ± 2.09 (47.9 mg / dL) for the RWD group. It was possible that the blood ethanol concentrations attained in this present study were not as high as the blood ethanol levels attained by ethanol vapour inhalation in Veatch's study.

A further complicating factor in this current experiment was the reversal of wake / sleep cycle during the course of the day observed in the CON group. From baseline recording to treatment day 8, control rats exhibited the expected patterns of vigilance states i.e. low activity levels during the dark phase. However, by treatment day 11 onwards, control animals unexpectedly exhibited lower levels of wakefulness during the dark phase in comparison to SWD and RWD rats.

One plausible explanation for this reversal of circadian rhythm in control animals could be attributed to the manner in which the animals were fed liquid diet. All treatment groups were given fresh liquid diet every day at 09:00 h. Rats which received ethanol liquid diet were provided with an excess amount to ensure they were not deprived of ethanol, thus avoiding the experience of an unscheduled withdrawal episode. The control group however, were provided with a daily amount of control liquid diet, based on the mean amount of ethanol liquid diet consumed by ethanol-treated rats, experiments conducted using automated liquid diet monitors revealed that control rats consumed all the liquid diet within the first 6 hours of administration. Hence, by the onset of the dark phase (19:00 h) there was no remaining liquid diet to be consumed and it was probable that control rats spent much of the dark phase in food deprivation, which may explain the more pronounced reversal in circadian patterns in the control rats compared to the ethanol-fed rats.

One distinctive feature of the circadian rhythm which arose during the course of chronic ethanol treatment in the current study was a "flattening out" of the WAKE curves in the SWD and RWD groups compared to the CON group, which demonstrated a distinct circadian rhythm, even when CON rats' circadian rhythm has been reversed. This result was interesting as it indicates that chronic ethanol consumption alters sleep architecture and rats' normal

ratio of wake: sleep. This may be a factor which contributes to the withdrawal syndrome and by post withdrawal day 3, the SWD and RWD groups appear to be re-establishing a circadian rhythm in their wakefulness (see **figure 2.9A**).

Radio-telemetric measurements of activity and core body temperature did not find any significant group differences as a result of ethanol consumption or ethanol withdrawal. However all rats exhibited the highest activity levels at 4pm-7pm which coincided with the onset of the dark phase particularly on day 11 (see **figure 2.10c**), the day after RWD rats had experienced a first intermediate withdrawal episode, on during which the ethanol-fed rats showed attenuated activity levels compared with the control rats. This effect of ethanol could not be attributed solely to the effects of the first withdrawal episode as there was no significant difference between the SWD and RWD groups and may therefore, be due to a general sedative effect of chronic ethanol consumption. This effect however, did not prove to be long lasting as by day 18 (see **figure 2.10d**), there were no significant differences between the treatment groups at any time bin during the course of the day. Locomotor activity has been reported to increase in the acute stages of ethanol withdrawal (Spanagel, Putzke et al. 1996). The effects of ethanol on locomotor activity has been found to be dose dependent, with low doses inducing hyperactivity, which is especially prevalent in mice (Cohen, Perrault et al. 1997) that sometimes sensitises upon repeated ethanol exposure (Hoshaw and Lewis 2001). However as the ethanol dose increases, the sedative effects of ethanol start to predominate, which leads to attenuated activity. The disparity between the findings on activity levels in this present study and those of Spanagel's study possibly lie in the different ethanol administration regimes as data from both studies were attained using similar radio-telemetric methods. Spanagel et al's study administered ethanol for a total of 7 days, during which the animals' drinking water was replaced by 20% (v/v) alcohol solution as their sole drinking fluid but animals were also provided with *ad libitum* access to standard rat chow, whereas in this current study, rats were provided with 7% ethanol liquid diet and *ad libitum* access to drinking water for a total treatment period of 24-30 days.

Acute injections of ethanol (1.5 g/kg i.p.) have been reported to produce significant decreases in body temperature in rats (Silveri and Spear 2000), an effect which reverses in withdrawal stages as ethanol withdrawal has been shown to lead to increased body temperature (Brick and Pohorecky 1977; Brick and Pohorecky 1983) possibly as a indicator of the development of tolerance. However, it is important to note that in the aforementioned studies, body temperatures were measured using rectal probes, the method of which could have resulted in sufficient stress-induced hyperthermia (Briese and Cabanac 1991; Oka, Oka et al. 2001; Olivier,

Zethof et al. 2003) to confound the interpretation of potential differences in body temperatures related to withdrawal. Furthermore, acute intraperitoneal injections of ethanol at 1.5 g/kg would result in considerably higher blood ethanol concentrations compared to that attained by consumption of 7% ethanol liquid diet. In this present study, body temperature and activity were monitored with the use of implanted telemetry probes, which were conducted in the home cage in singly housed animals, a condition which may also have impacted on the experiment as rats may find isolation particularly stressful (Stone and Quartermain 1997). Hence, the current experiment may not have detected any significant differences in body temperature between the treatment groups due to the development of tolerance to chronic ethanol effects on body temperature and the high stability of body temperatures may have been attributed to the assessment of this measure in the home cage by radio-telemetry measures rather than by rectal probe.

Blood ethanol concentrations of SWD and RWD groups on corresponding days appear to follow a similar pattern suggesting that no difference exists between the blood ethanol levels of these treatment groups. However, it was also possible that blood samples taken from the rat tail vein poorly represents trunk blood. Blood can be sampled from rats using different techniques and although the method of blood sampling can also affect the outcome of blood analysis, a study by Herck and associates reported that there were no significant differences in blood constituents between blood sampling from the retro-orbital plexus, the saphenous vein and the tail vein (Van Herck, Baumans et al. 2001) although the haemoglobin found in tail vein blood was lower than in orbital blood. Additionally, a study by Vahl and colleagues reported no significant differences in the stress response profile of tail vein blood sampling and catheterisation although authors stress the importance of limiting sampling time to 2-3 minutes so as not to alter the HPA axis stress response (Vahl, Ulrich-Lai et al. 2005).

Measurements of post-withdrawal food and water intake found no significant effect of repeated ethanol withdrawal on food intake, however, SWD rats drank more water after withdrawal compared to CON rats indicating an effect of a first withdrawal. However, the difference in water intake between SWD and RWD did not reach significance. Food intake has been reported to initially increase but then decrease significantly below basal food intake one day after ethanol withdrawal (Spanagel, Putzke et al. 1996). Post withdrawal food intake in the current experiment also followed a similar pattern to those reported by Spanagel, however there was no significant difference between the groups which suggests that the pattern of post-withdrawal food intake may be more plausibly attributed to the switch from liquid diet to standard rat chow. Although from a literature search, there appear to be no available data on

the behavioural effects of changing from liquid diet to rat chow, we postulate that this dietary switch from liquid to solid food may produce general behavioural effects in food intake, as observed across the treatment groups (See **Figure 2.14**).

Post withdrawal water intake increased significantly as a result of alcohol withdrawal (as observed in both SWD and RWD rats) but no increased withdrawal severity was observed as a consequence of repeated ethanol withdrawal. This withdrawal symptom was not long lasting as the effect had dissipated by post withdrawal day 4, indicating ethanol withdrawal effects on water intake occurred in short term withdrawal and did not persist into abstinence. Although animal studies investigating the post withdrawal effects of chronic ethanol treatment on food and water intake are not well documented, one possible interpretation of the current findings is the alcohol-fed rats are compensating for the dehydrating effects of chronic ethanol consumption.

Results from this present investigation suggest that rats that have experienced a single ethanol withdrawal produced greater withdrawal severity as measured by post withdrawal food and water intake. Furthermore RWD rats appeared to overcome withdrawal symptoms faster (see **Figure 2.15**), as RWD rats' water intake was similar to control rats but SWD rats showed marginal elevation of water intake compared with the other treatment groups. This "toughening up" effect may reflect a similar phenomenon to the reduced blood cortisol levels seen in rats that have undergone repeated ethanol withdrawal relative to single ethanol withdrawal rats (Borlikova, Le Merrer et al. 2006).

In conclusion, our model of repeated ethanol withdrawal produced withdrawal symptoms in post withdrawal food and water intake, similar to those reported in previous rodent studies. Furthermore, it appears from the current set of experiments that single withdrawal rats may suffer from more prolonged withdrawal symptoms in comparison with RWD rats that appear to overcome withdrawal symptoms more rapidly that SWD rats, based on post withdrawal water intake. Additionally, both SWD and RWD rats showed disruption of sleep architecture revealing that chronic ethanol consumption led to disruptions in EEG / EMG measures from which sleep architecture was deduced. However, repeated ethanol withdrawal effects were not evident in measures of body temperature and activity, which demonstrates that although certain withdrawal symptoms can be reproduced in rats using our chronic ethanol administration protocol, other withdrawal symptoms may be more subtle and hence may require a higher blood ethanol concentration in order to model the withdrawal symptom.

Chapter 3

The effects of repeated ethanol withdrawal on immediate early gene c-fos and zif268 expression in the rat prefrontal cortex

3.1. Introduction:

Repeated episodes of withdrawal from alcohol abuse have been established to lead to an increased risk of withdrawal-induced seizures (Gross, Rosenblatt et al. 1972; Ballenger and Post 1978; Brown, Anton et al. 1988; Lechtenberg and Worner 1991; Lechtenberg and Worner 1992; Booth and Blow 1993). More recently, cognitive deficits have been reported in patients that have previous experience of withdrawal episodes (Duka, Gentry et al. 2004). Severe chronic alcohol dependence has been consistently linked to cognitive impairments such as cognitive flexibility, problem solving, decision making, risky behaviour and other cognitive functions, (Bechara, Dolan et al. 2001; Fein, Klein et al. 2004; Noel, Bechara et al. 2007; Glass, Buu et al. 2009). These cognitive functions are all associated with frontal lobe functions (Fein, Bachman et al. 1990; Chanraud, Martelli et al. 2007; Noel, Bechara et al. 2007).

The prefrontal cortex is the association area of the frontal lobes, which is of particular importance as it receives connections from all sensory modalities (Martin 2006). The prefrontal cortex which forms part of the neocortex, is the last region of the human brain to mature (Goldman-Rakic 1987) and may thus be especially susceptible to insult in late adolescence. The prefrontal cortex has multiple functions and when properly functioning, allows individuals to use past experience and knowledge to make sense of current behaviour and guide future responses (Stuss 1992; Stuss and Alexander 2000). The human prefrontal cortex is divided into 3 interacting subcircuits: dorsolateral, orbitofrontal and anterior cingulate cortices (Alexander, DeLong et al. 1986). The dorsolateral prefrontal circuit mediates executive function, including the attentional control, and problem solving (Cummings 1993; Stuss and Alexander 2000; Abe and Hanakawa 2009). Executive functions include maintaining divided and sustained attention, sequencing, set shifting and cognitive flexibility, amongst other functions (Luria 1973) .The medial prefrontal cortex is essential for feedback monitoring and motivation and lesions to this prefrontal area lead to profound apathy (Bonelli and

Cummings 2007). The orbitofrontal cortex, in conjunction with the dorsolateral prefrontal cortex, is responsible for regulation of behaviour due to its unique capacity to maintain and integrate sensory, affective and associative information (Carmichael and Price 1995; Carmichael and Price 1995). These functions allow an individual to recognise expected outcomes and thus use information to guide behaviour (Schoenbaum, Roesch et al. 2006).

Researchers have reported that alterations in prefrontal cortical activity lead to reductions in behavioural control and decision-making (Jentsch and Taylor 1999; Franklin, Acton et al. 2002; Goldstein and Volkow 2002). Much attention has been focused on the orbitofrontal cortex and its role in drug addiction. The orbitofrontal cortex is thought to be involved in craving. Evidence for this derives from functional imaging studies that highlight orbitofrontal cortex activation when addicts are exposed to drug-associated stimuli that elicit drug craving, (Grant, London et al. 1996; Childress, Mozley et al. 1999; Garavan, Pankiewicz et al. 2000; Goldstein and Volkow 2002; Goldstein, Tomasi et al. 2007). Interestingly, there is evidence that compulsive drug seeking behaviour exhibited by drug addicts and its persistence in the face of negative consequences is similar to the behaviour of individuals with orbitofrontal damage or dysfunction (Rogers, Everitt et al. 1999; Bechara and Van Der Linden 2005). Additional support for the role of the orbitofrontal cortex in drug addiction comes from further imaging studies which have reported persistent metabolic and neurochemical changes in the orbitofrontal cortex of drug addicts, including abstinent drug addicts, which suggests that long term drug abuse may lead to functional changes in the prefrontal cortex which contribute to the development of drug addiction (Goldstein and Volkow 2002; Volkow, Fowler et al. 2003). Human studies investigating behaviours of patients with lesions to the orbitofrontal cortex have reported impulsive or perseverative behaviours (Bechara, Damasio et al. 1994; Rolls, Hornak et al. 1994; Berlin, Rolls et al. 2004). Studies in animals also appear to support the link between dysfunction of the orbitofrontal cortex and compulsive behaviours including substance abuse (Bechara and Van Der Linden 2005; Schoenbaum, Roesch et al. 2006). It is important to note that although the prefrontal cortex may not be directly involved in the brain mechanisms which control drug seeking and drug taking behaviour, the changes that

occur as a consequence of drug taking lead to dysfunction in prefrontal cortical areas that contribute to the compulsive aspect of drug addiction (Everitt and Robbins 2005). Although the biological mechanisms underlying addiction have been explored in much detail, the cognitive aspects of addiction have received little research focus despite converging evidence suggesting that disruption or dysfunction of cognitive control is a hallmark of addiction (Ersche, Fletcher et al. 2005; Garavan and Stout 2005; Wilson, Sayette et al. 2007). Furthermore, clinical studies have reported similarities between drug addicts and patients with prefrontal cortical damage. For instance, damage to the orbitofrontal cortex (Berlin, Rolls et al. 2004) but not the ventromedial frontal lobe (Fellows and Farah 2005), increases impulsive choice, the tendency to choose smaller immediate rewards over delayed larger rewards. This evidence is further supported by studies of rat lesions to the orbitofrontal cortex (Mobini, Body et al. 2002; Rudebeck, Walton et al. 2006). This preference for immediate rewards may be perceived as a form of impulsivity (Evenden 1999), an important phenotype relating to the neural bases of addiction (Reynolds 2006). Injury to the prefrontal cortex does not affect intelligence, memory or other cognitive functions but affect and social behaviour changes (Bechara and Van Der Linden 2005). This finding is of particular importance as it has been reported that patients with previous history of multiple detoxifications show increased negative emotional sensitivity (Duka, Townshend et al. 2002) and in rats that have previous withdrawal experience, there are deficits in conditioned fear (Stephens, Brown et al. 2001; Stephens, Ripley et al. 2005; Townshend and Duka 2005). This evidence points towards a role for the prefrontal cortex in addiction, possibly reflecting damage arising from brain overactivation during alcohol withdrawal due to kindling-like processes in the limbic brain regions. Due to the extensive neuronal connectivity of the PFC to the limbic system, including the amygdala, we would expect to observe activation of prefrontal cortical areas in response to repeated episodes of ethanol withdrawal in the rat.

Although this increasing evidence of impaired frontal function in alcoholics, it is not clear whether such dysfunction existed prior to alcohol abuse or whether it is a consequence of abuse; and if the latter, what mechanisms might be involved in PFC dysfunction. We postulate that PFC activation will be reflected by the induction of the

immediate early gene c-*F*os and neuronal plasticity in PFC areas will be observed by the induction of another immediate early gene zif268.

Immediate early genes are characterised by their rapid and transient induction in response to a wide range of different stimuli (Morgan and Curran 1991). Immediate early genes encode proteins which carry out their functions as transcription factors in order to mediate, regulate and control the expression of other target genes which may be involved in cellular growth and differentiation. The expression of c-*F*os is induced in the brain after the occurrence of electrically and chemically induced seizures (Morgan, Cohen et al. 1987; Sagar, Sharp et al. 1988) , in neurones following peripheral somatosensory stimulation (Daval, Nakajima et al. 1989) and in cultured cells treated with glutamate (Szekely, Barbaccia et al. 1987). The reports of these investigations led to the suggestion that c-*F*os can be used an indicator of increased physiological activity of individual cells. Immediate early gene expression has been used to assess the pattern of neuronal activation in rodents in a similar manner to that of functional neuroimaging in clinical studies. Studies of immediate early gene expression in rodents with previous experience of ethanol withdrawal have found increased c-fos expression (Dave, Tabakoff et al. 1990; Morgan, Nadi et al. 1992; Matsumoto, Davidson et al. 1993; Wilce, Beckmann et al. 1994; Knapp, Duncan et al. 1998; Moy, Knapp et al. 2000; Olive, Mehmert et al. 2001; Borlikova, Le Merrer et al. 2006).

Immediate early gene Zif268 (also known as KROX-24, egr-1, tis-8 and NGFI-A) encodes a zinc finger protein which binds to another sequence (Christy, Lau et al. 1988) which in doing so regulates the expression of a different set of target genes. Zif268 has been proposed to be a key component of a cascade of events involved in cortical plasticity (Wallace, Withers et al. 1995). Zif268 expression also appears to be dependent on NMDA receptor activation (Cole, Saffen et al. 1989) and is crucial for the maintenance of late phase long term potentiation (LTP) (Jones, Errington et al. 2001; Ko, Ao et al. 2005). Studies from this laboratory (Borlikova, Le Merrer et al. 2006) have found Zif268 expression was increased as a consequence of single episode of ethanol withdrawal in the central amygdala but Zif268 expression was not increased as a result of repeated ethanol withdrawal, indicating that repeated episodes of ethanol withdrawal reduce neuronal plasticity, despite increasing neuronal activation in the limbic brain areas.

This present experiment was conducted in an attempt to identify the prefrontal brain areas influenced by repeated ethanol withdrawal and the ability of the withdrawal process to affect the expression of immediate early genes that represent expression of both neuronal activity and neuronal plasticity, c-*f*os and zif268, respectively. Immediate early genes c-fos and zif268 expression was assessed in prefrontal cortical areas.

3.2 Materials and Methods:

3.2.1. Subjects:

Thirty-six male Lister hooded rats, *n* = 12 (190 – 230g at the beginning of the experiment, Harlan, UK) were used. All animals were pair-housed and maintained on a 12-h light/dark cycle (lights off at 19:00h; temperature 21± 2°C; humidity 50±10%) with *ad libitum* access to water and rodent chow (Bekay Feeds, Hull, UK) for seven days' acclimatisation. All procedures were carried out in accordance with UK Animals (Scientific Procedures) Act 1986, following Home Office approval.

3.2.2. Chronic ethanol liquid diet treatment:

Chronic ethanol treatment administered was identical to methods described in chapter 2.

3.2.3. Histology of immediate early genes C-fos and Zif268 expression in prefrontal areas.

In order to investigate immediate early gene c-Fos and Zif268 expression by repeated ethanol withdrawal, rats were sacrificed for perfusion. Eight hours after the final withdrawal, rats were deeply anaesthetised using sodium pentobarbital (Euthatal, 60 mg/kg i.p.) and transcardially perfused with 0.1 M phosphate buffered saline (PBS) (Phosphate buffer; disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate in distilled water) and then with fixative 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were excised and placed in 4% paraformaldehyde in 0.1 M PBS for 24 hours, and then cryoprotected in 30% sucrose (in PBS) for a futher 24 – 48 hours before freezing in isopentane (2-methylbutane, Sigma Aldrich) at -50°C and stored at -80°C.

Brains were coronally sectioned at 30 µm using a freezing microtome. Sections were collected in PBS (alternate sections were collected for conventional histology to confirm brain anatomy), incubated with 0.3% H_2O_2 for 10 minutes and washed in PBS. After 1 hour incubation with 1.5% normal goat serum (NGS, Vector Laboratories), sections were washed in PBS and incubated overnight in PBS containing 0.5% NGS, 0.02% sodium azide (Sigma Aldrich), 0.3% Triton X-100 (Sigma Aldrich) and 1:10,000 c-*fos* primary antibody (rabbit polyclonal serum, Oncogene, Ab-5) or 1:1,600 zif268 primary antibody (Egr-1 (588), rabbit polyclonal serum, Santa Cruz Biotechnology, sc-110). The next day, sections were washed and incubated for 1 hour in PBS containing 1.5% NGS and 1:300 biotinylated anti-rabbit secondary antibody (Vector Laboratories, BA-1000). After washing, the sections were incubated in PBS containing 1:1,000 avidin-biotinylated-horseradish peroxidise complex (Vectastain ABC kit Elite, Vector Laboratories.) The reaction was visualised using a standard glucose oxidase-3,3-diaminobenzidine method. The reaction was terminated by extensive washing with PBS.

Fos-positive nuclei were quantified from images of sections captured using an AxioCam HRc digital camera mounted in a Zeiss Akioskop 2 plus microscope (Carl Zeiss, UK) using Axio Vision 3.1 software (Imaging associates, Bicester, UK) with x100 magnification. The regions studied were the prelimbic cortex (500 μm^2), lateral orbital cortex (500 μm^2), medial orbital cortex (500 μm^2), ventral orbital cortex (500 μm^2), infralimbic cortex (500 μm^2), anterior cingulate cortex (500 μm^2), and agranular insula (500 μm^2).

Cell counting was conducted using Scion Image for Windows (Scion Corporation, MD, USA). The locations of the brain areas used for quantifying Fos-positive or zif268-positive nuclei were taken from the atlas of Paxinos and Watson (1998) using conventionally stained neighbouring sections as a guide to accurate brain locations. Counts were taken bilaterally in two sections for each brain region and the mean of these four values was taken as the number of nuclei for that brain region for that animal.

3.3. Results:

There was found to be no significant difference in ethanol consumption between the single and repeated ethanol withdrawal. Areas of the prefrontal cortex in which immunostaining was assessed are shown in **Fig 3.3.** Mean numbers of Fos-positive nuclei for the different prefrontal cortical areas are shown in **Fig 3.5.** Typical photomicrographs of c-*fos* expression in the prelimbic cortex are shown in **Fig 3.2.**

Mean numbers of zif-positive nuclei for the different prefrontal cortical areas are shown in **Fig 3.6.** Typical photomicrographs of zif expression in the prelimbic cortex are shown in **Fig 3.3.**

With regard to c-*fos* expression, repeated ethanol withdrawal induced higher levels of neuronal activation in comparison with rats in the control group and rats that had undergone a single withdrawal from ethanol. Repeatedly withdrawn rats showed significantly higher c-*fos* expression in prelimbic cortex, anterior cingulate cortex, ventral orbital cortex, lateral orbital cortex and the agranular insula cortex than CON and SWD rats. These data demonstrate that the process of repeated ethanol withdrawal activated the PFC as a whole with no specific differentiation between the areas of the PFC.

Zif268 expression was found to be significantly higher in the prelimbic cortex of repeatedly withdrawn rats but zif268 expression was not found to be significantly higher in the other PFC regions examined.

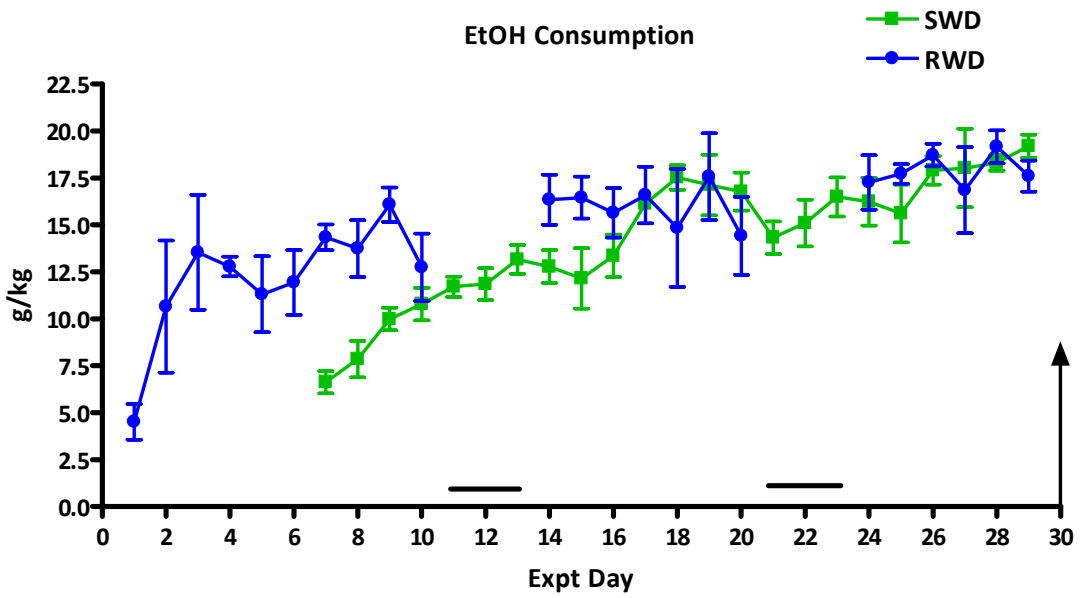


Fig 3.1: Ethanol consumption expressed as g per kg of body weight per treatment day. Data collected from measurement of change in bottle weight in a 24-hour period. Data represent mean ± sem. Arrow indicates the final withdrawal; intermediate withdrawal episodes in RWD group took place on experimental days 11 – 13 and 21 – 23. There was no overall difference between the groups in consumption (p = 0.701). SWD and RWD groups consumed an average of 14 g/kg.

As illustrated by **figure 3.1**, there was no significant difference in ethanol consumption between SWD and RWD groups during chronic ethanol treatment ($F_{1, 14} = 1.59$, $p = 0.23$, $\varepsilon = 0.32$).

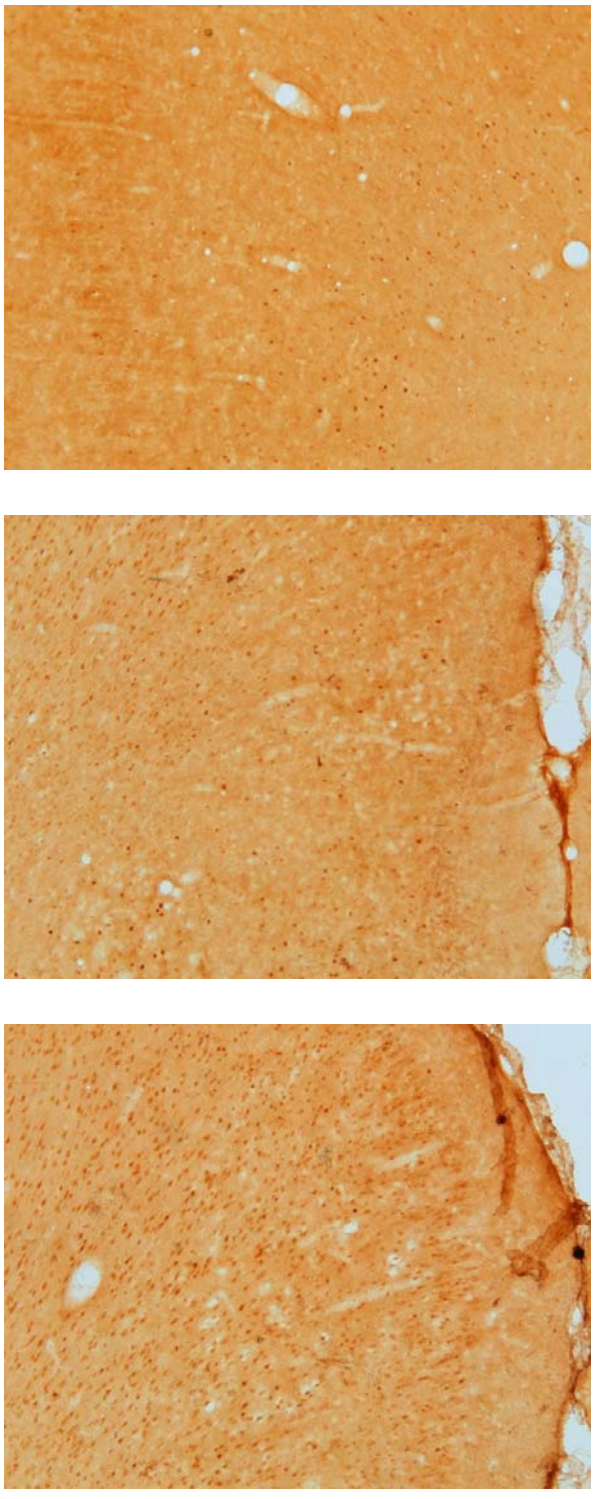


Figure 3.2: Representative photomicrographs (magnification x 100) of the prelimbic cortex of the rat prefrontal cortex illustrating immunostaining of c-*fos* in the 3 treatment groups. Controls (A), SWD (B) and RWD (C).

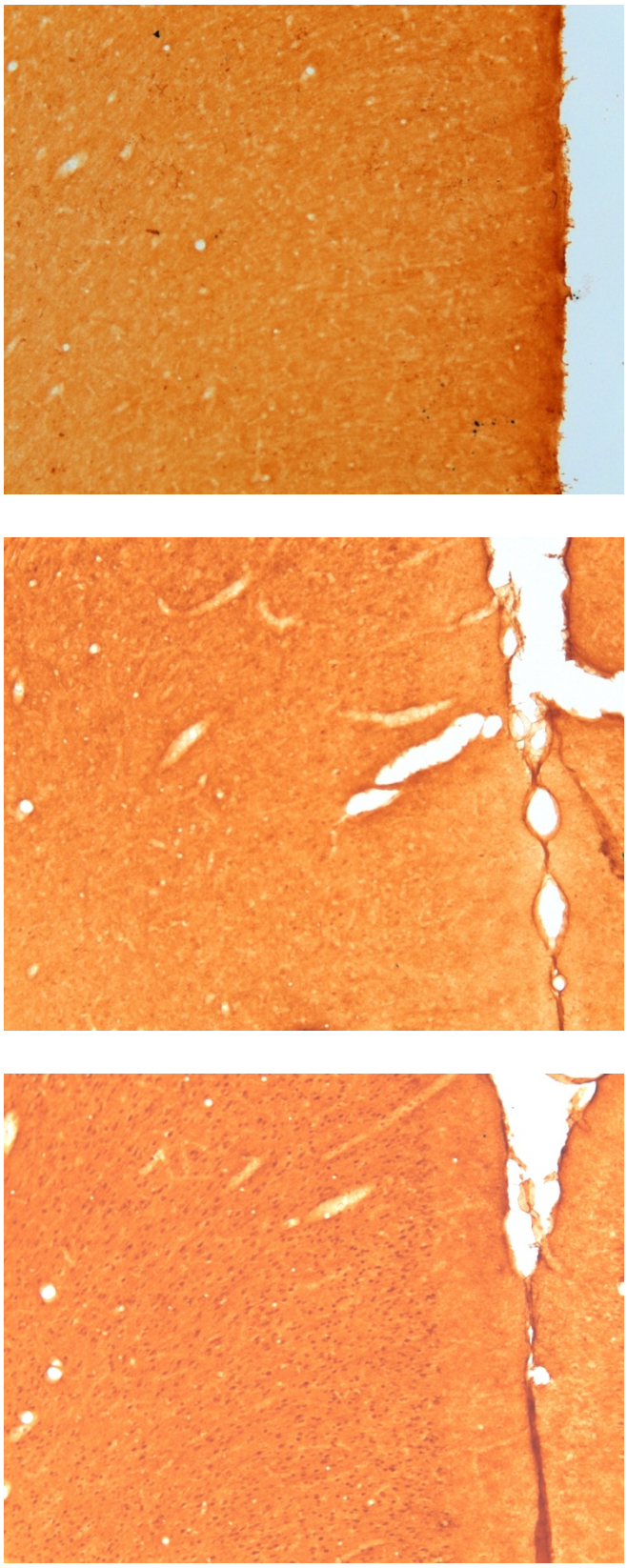


Figure 3.3: Representative photomicrographs (magnification x 100) of the prelimbic cortex of the rat prefrontal cortex illustrating immunostaining of Zif268 in the 3 treatment groups. Controls (D), SWD (E) and RWD (F).

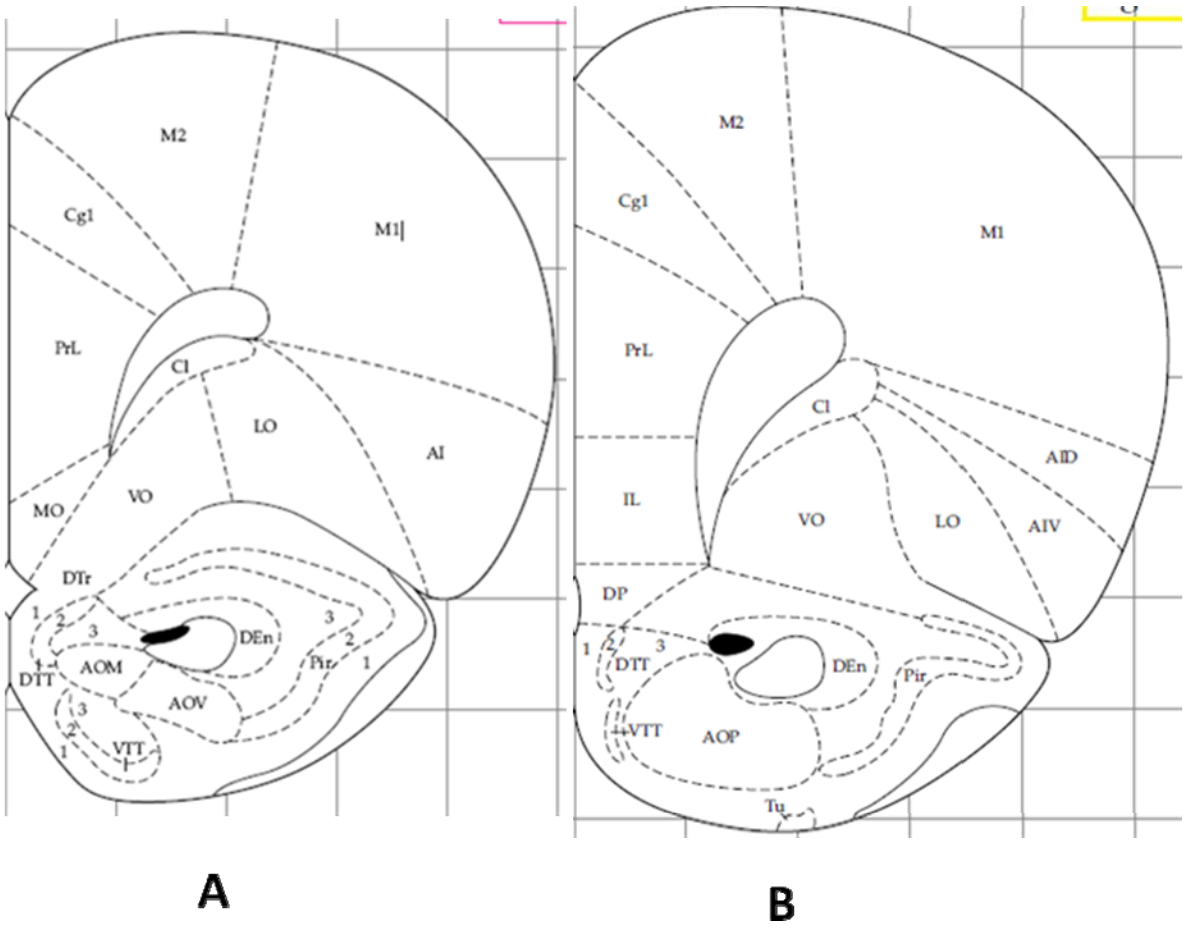


Figure 3.4: Schematic diagrams, adapted from the atlas of Paxinos and Watson (1998) showing areas of the rat prefrontal cortex in which c-*fos* and Zif268 expression was quantified. Measurements from the bregma in (A) + 3.70 mm and (B) +3.20 mm. Cg1 – *anterior cingulate cortex,* PrL – *Prelimbic cortex,* IL – *infralimbic cortex,* MO – *medial orbital cortex,* VO – *ventral orbital cortex,* LO – *lateral orbital cortex,* AI – *agranular insula cortex,* AID – *dorsal agranular insula cortex,* AIV – *ventral agranular insula cortex.*

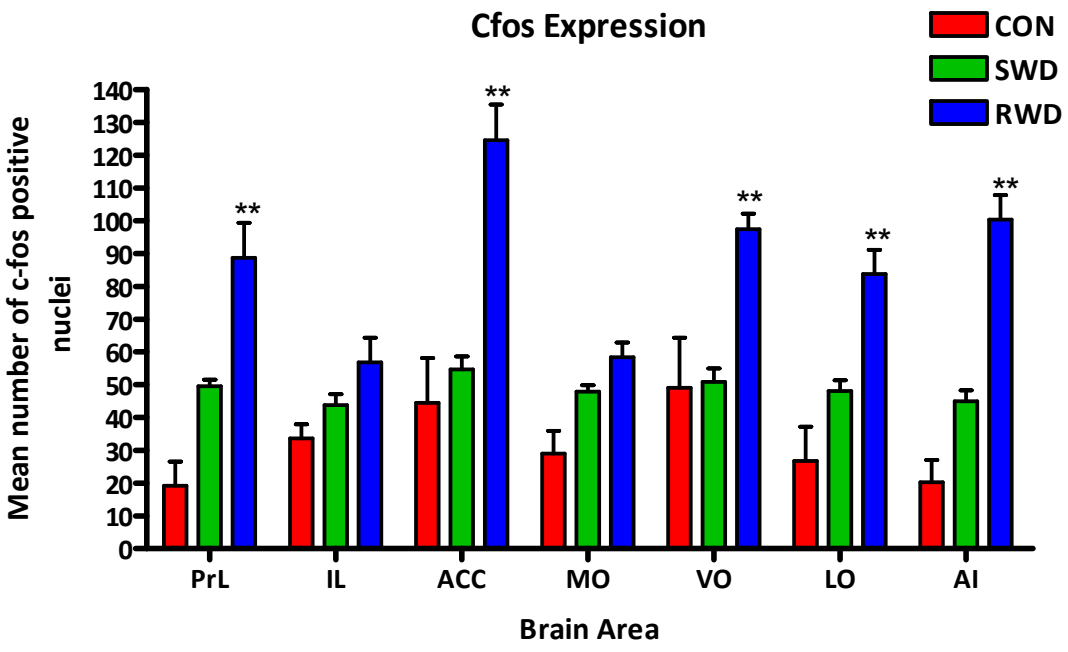


Fig 3.5: C-*fos* immunoreactivity in the prefrontal cortex. Mean ± SEM number of c-fos positive nuclei in prefrontal cortical areas after exposure to chronic ethanol treatment. CON group = red bars, SWD group = green bars and RWD group = blue bars. RWD rats showed significantly higher c-fos expression compared with CON and SWD rats.

As illustrated by **Figure 3.5**, there was a significant main effect of brain area on c-Fos expression, ($F_{6, 72}$ =3.49, p = 0.031). There was a significant group difference in c-Fos expression ($F_{2, 12}$ =4.79, p = 0.03). C-Fos expression in rats that had experienced repeated ethanol withdrawal was found to be significantly higher compared to both CON (p = 0.016) and SWD (p = 0.031) groups. There was no significant difference in the c-Fos expression between CON and SWD groups (p = 0.38). This indicates that c-Fos expression is increased in prefrontal cortical areas after repeated, but not after single withdrawal from ethanol. Further analysis revealed that in the prelimbic cortex , anterior cingulate cortex, ventral orbital cortex, lateral orbital cortex and agranular insular cortex, repeated ethanol withdrawal induced significantly higher levels of c-fos expression compared to both CON and SWD groups (all at p<0.05). However there were found to be no significant group differences in c-Fos expression in infralimbic cortex ($F_{2, 14}$ =0.597, p = 0.57) and medial orbital cortex ($F_{2, 15}$ = 1.38, p = 0.29).

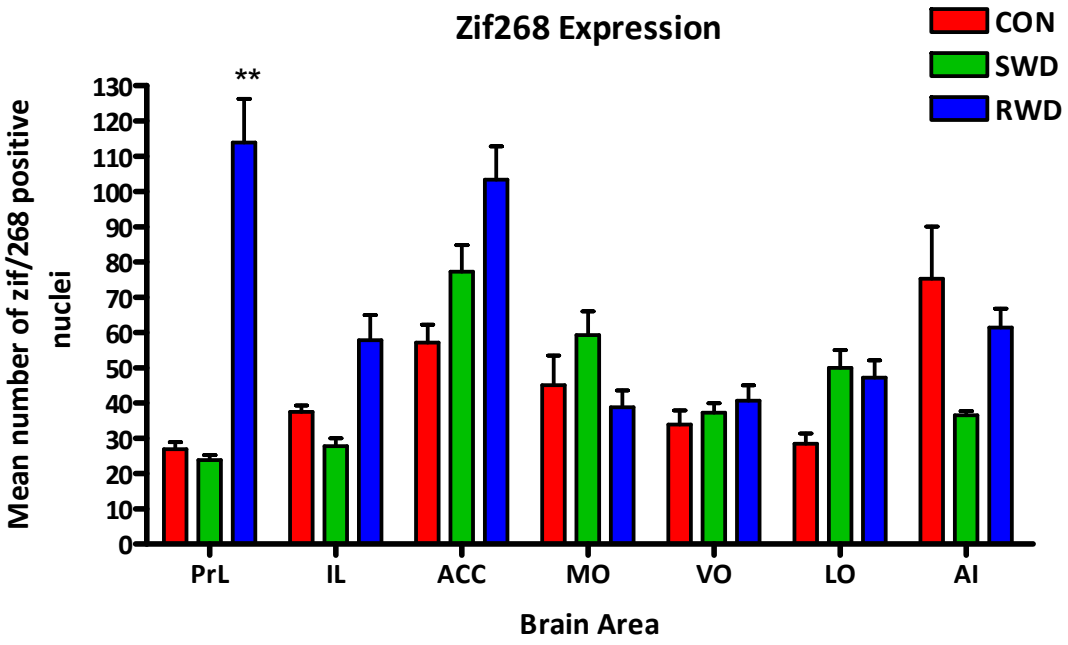


Fig 3.6: Zif268 immunoreactivity in the prefrontal cortex. Mean ± SEM number of Zif268 positive nuclei in prefrontal cortical areas after exposure to chronic ethanol treatment. CON group = red bars, SWD group = green bars and RWD group = blue bars. RWD rats showed significantly higher zif268 expression in the PrL compared to CON and SWD rats.

As illustrated by figure 3.6, there was a significant main effect of brain area on the level of zif268 expression ($F_{6, 66} = 3.17$, $p = 0.009$). There was also a group x brain area interaction ($F_{12, 66} = 2.53$, $p = 0.008$). Repeated ethanol withdrawal produced higher levels of Zif268 expression compared with CON ($p = 0.056$) and SWD ($p = 0.054$) groups which approached significance. Further analysis revealed that there were significant group effects in Zif268 expression. Repeated ethanol withdrawal gave rise to higher levels of Zif268 expression in the prelimbic cortex compared with CON ($p = 0.004$) and SWD ($p = 0.002$) groups. There were no significant group differences in zif268 expression in agranular insula cortex ($F_{2, 13} = 1.31$, $p = 0.31$) **, lateral orbital cortex ($F_{2, 15} = 0.67$, $p = 0.53$), ventral orbital cortex ($F_{2, 15} = 0.167$, $p = 0.85$), medial orbital cortex ($F_{2, 13} = 0.51$, $p = 0.63$), anterior cingulate cortex ($F_{2, 13} = 1.50$, $p = 0.26$), infralimbic cortex ($F_{2, 13} = 1.91$, $p = 0.19$). It must be noted that varying degrees of freedom in these results, was attributed to missing sections in which cell counts could not be completed.

3.4 Discussion:

The results of the present study demonstrate the expression of immediate early genes c-*F*os and Zif268 in the rat brain following repeated ethanol withdrawal and the consequences of multiple ethanol withdrawal experiences compared to a single withdrawal from ethanol. Repeated ethanol withdrawal induced significantly higher levels of c-*f*os expression than single withdrawal or control group in prefrontal cortical areas in the rat. Rats with previous experience of ethanol withdrawal showed significantly higher levels of zif268 expression in the prelimbic cortex. Although rats that experienced repeated ethanol withdrawal show trends towards higher levels of zif268 expression in the infralimbic cortex and the anterior cingulate cortex, these did not reach significance. Previous experiments in this laboratory investigating immediate early gene expression c-*F*os and zif268 in repeated ethanol withdrawal have looked into the amygdala, hippocampus, nucleus accumbens and periaqueductal grey and the role of these areas in withdrawal-induced anxiety (Borlikova, Le Merrer et al. 2006). Borlikova and colleagues reported a significant increase in *F*os expression following repeated withdrawal but not single withdrawal in the central and the basolateral nuclei of the amygdala, in the CA3 field of the hippocampus, the nucleus accumbens core and the dorsolateral PAG. Expression of immediate early gene zif268 was increased after a single withdrawal in the central nucleus of the amygdala of the SWD group only, whereas in RWD rats, zif268 expression was similar to the control group (Borlikova, Le Merrer et al. 2006).

The findings of this present study of c-*F*os and Zif268 expression in the prefrontal cortex indicates that neuronal activation occurred as a result of repeated episodes of ethanol withdrawal, and neuronal plasticity occurred only in the prelimbic cortex as a result of repeated ethanol withdrawal. There were significant increases in c-fos expression in prelimbic cortex, anterior cingulate cortex, ventral orbital cortex, lateral orbital cortex and agranular insula cortex. This finding indicates that repeated ethanol withdrawal gives rise to a general increase in neuronal activation in most prefrontal areas investigated. Thus, there was no specific area of the prefrontal cortex that was differentially activated over another. It was found however, that single and repeated ethanol withdrawal differentially activated c-fos expression. Neuronal plasticity as

measured by zif268 expression, increased solely in the prelimbic cortex as a consequence of repeated ethanol withdrawal. Borlikova et al (Borlikova, Le Merrer et al. 2006) did not find increased plasticity in the limbic brain regions, with the exception of the central nucleus of the amygdala, as a consequence of repeated ethanol withdrawal, and on this basis, it is perhaps surprising that prelimbic cortex displayed evidence of increased plasticity after repeated cycles of ethanol withdrawal, given that the prefrontal cortex has reciprocal connections with limbic brain regions, particularly the amygdala. However, it is possible that the prelimbic cortex is more susceptible to the effects of repeated ethanol withdrawal than the other parts of the prefrontal cortex. The sensitivity of the frontal lobes to the chronic effects of alcohol is supported by post-mortem studies which have reported that alcoholics showed a significant loss of brain tissue, especially from the white matter of the cerebral hemispheres (Harper, Kril et al. 1985).

The prefrontal cortex of normal individuals has long been implicated in storage and executive, specific mnemonic and working memory components, of goal-directed actions in humans and primates (Goldman-Rakic 1987; Kimberg and Farah 1993; Stuss and Alexander 2000; Fuster 2001; Fuster 2002). Many studies have correlated the prefrontal cortex with various cognitive functions including action selection (Rowe, Toni et al. 2000), planning (Baker, Rogers et al. 1996; Rainer, Rao et al. 1999), and selective attention (Robbins 1997; Wall and Messier 2001). The prelimbic cortex and the infralimbic cortex combined comprise the medial prefrontal cortex. Although the prelimbic and infralimbic cortices are closely connected, there was no significant difference in neuronal activation between the groups in the infralimbic cortex. Interestingly, the prelimbic cortex has now been reported to play a role in the expression of fear which is interesting, as studies in this laboratory have reported that repeated ethanol withdrawal leads to impaired fear learning which may suggest prelimbic dysfunction.

The prelimbic cortex sends robust projections to the basal nucleus of the amygdala (McDonald, Mascagni et al. 1996; Vertes 2004) a brain area which is necessary for both the acquisition and expression of fear responses. In addition, the neurones in the prelimbic cortex show fear-related increases in activity (Baeg, Kim et al. 2001;

Gilmartin and McEchron 2005; Laviolette, Lipski et al. 2005). Although injury to the prefrontal cortex does not affect intelligence or memory, injury can alter affect and social behaviour (Bechara and Van Der Linden 2005). Clinical studies have found that patients with prior history of ethanol withdrawals show increased negative emotional sensitivity (Duka, Townshend et al. 2002), an observation which is supported by findings of deficits of conditioned fear in rats (Stephens, Brown et al. 2001; Stephens, Ripley et al. 2005; Townshend and Duka 2005). These deficits in learning about aversive conditioned stimuli as a consequence of repeated withdrawal point toward disrupted amygdala function as a result of the withdrawal process. Studies in rats using tetrodotoxin to inactivate the prelimbic cortex have reported that prelimbic activity is crucial for the expression but not acquisition of learned fear (Corcoran and Quirk 2007).

The anterior cingulate cortex has received much research focus and is thought to be involved in impulsivity. In a review by Volkow and colleagues (Volkow, Fowler et al. 2003), authors identified the anterior cingulate cortex and the orbitofrontal cortex as the prefrontal areas crucially involved in cognitive and behavioural flexibility in the addiction process. It has been suggested that the behavioural decision that drug users make on whether or not to continue drug use is partly mediated by prefrontal cortical areas which are involved in inhibitory control. With increased drug use, the neural circuits in the prefrontal cortex that regulate drive, motivation and reward weakens, allowing drug seeking and drug taking to become increasingly controlled by subcortical brain circuits regulating motivation, reward and learning and memory, leading to increasingly compulsive behaviours which are characteristic of drug addiction (Volkow, Fowler et al. 2003). Rats with lesions to the anterior cingulate cortex have been found to over-respond to unrewarded stimuli (Bussey, Everitt et al. 1997; Parkinson, Willoughby et al. 2000) and to respond prematurely in situations where they are required to wait (Muir, Everitt et al. 1996). However rats with anterior cingulate cortex lesions did not exhibit performance deficits in delayed discounting task, a task which requires the subject to make a choice between a small immediate reward or a larger delayed reward (Cardinal, Pennicott et al. 2001). It is well established that motor impulsivity is dissociable from impulsive choice. The anterior cingulate cortex (amongst

other prefrontal structures) is linked to motor impulsivity (Muir, Everitt et al. 1996), but does not appear to mediate impulsive choice. This result might suggest the finding that repeatedly withdrawn rats demonstrated over-responding in a fixed interval operant task (Borlikova, Elbers et al. 2006) was attributable to altered function in the prelimbic cortex.

In humans, the orbitofrontal cortex sends projections to the core of the nucleus accumbens and is strongly implicated in the assessment of reward value. Lesions of the orbitofrontal cortex have been reported to induce impulsive choice by some laboratories (Mobini, Body et al. 2002) whereas others have found that rats with lesions to the OFC exhibited superior self control in comparison with sham rats (Winstanley, Theobald et al. 2004). It is possible that the discrepancies between these 2 findings of OFC function were possibly due to the time point at which training occurred. In Winstanley et al (2004), investigators trained rat subjects prior to lesioning, whereas in Mobini et al's study, training and testing occurred post-operatively. These findings suggest that c-*F*os activation in the rat orbitofrontal cortex found in the present study may predict that repeated withdrawn rats may assign more importance to rewards, as according to Mobini et al (2002), or have no effect on the subjects' perception of reward value, according to Winstanley et al (2004).

The agranular insula cortex also comprises part of the orbitofrontal cortex and plays an important role in the anticipation or expectancy of reward value in primates (Tremblay and Schultz 1999; Hikosaka and Watanabe 2000) and humans (DeCoteau, Kesner et al. 1997; Ragozzino and Kesner 1999; Di Pietro, Black et al. 2004). Hence it appears that the agranular insular portion of the orbitofrontal cortex may play a crucial role in mediating the anticipation of reward value measured across a delay between a response and a reward (Kesner and Gilbert 2007).

It is important to note at this stage that different studies investigating the effects of chronic ethanol treatment on immediate early gene expression have used different paradigms of ethanol exposure which may vary in duration of exposure and route of administration. Generally chronic ethanol treatment to rodents may be classified as a minimum of 14 days of ethanol exposure (Vilpoux, Warnault et al. 2009). In chronic

ethanol studies involving chronic ingestion for 6 weeks in adult male and female rats, there was no effect on c-*F*os mRNA as visualised by RT-PCR methods in the midbrain, cortex, brainstem and cerebellum (Nakahara, Hirano et al. 2002). Chronic exposure to ethanol vapour did not increase c-*F*os expression in the rat brain regions examined (Wilce, Le et al. 1993) which is supported by the lack of difference in c-fos expression in the SWD group and the CON group. However, withdrawal-induced c-*F*os expression in rodents, measured by immunohistochemistry or in situ hybridisation show increases in prefrontal cortex areas with different routes of administration, including intraperitoneal injections (4 g/kg i.p.) (Kozell, Hitzemann et al. 2005), an intragastric 15% ethanol based diet for 4 days, (Knapp, Duncan et al. 1998), 7% ethanol liquid diet as a sole food source for 14 days (Knapp, Duncan et al. 1998; Moy, Knapp et al. 2000), and for 30 days with 2 intermediate withdrawal episodes (Borlikova, Le Merrer et al. 2006) and a 20% ethanol solution as sole source of fluid for 14 days (Putzke, Spanagel et al. 1996). Hence, the findings of the present study provide further evidence of the sensitivity of the prefrontal cortex to ethanol withdrawal.

On the contrary, increased neuronal plasticity was only detected in the prelimbic cortex as a consequence of repeated ethanol withdrawal. One interpretation of this finding would be that the capacity for withdrawal-induced plasticity in prelimbic neurones increased following repeated ethanol withdrawal but were unaffected by a single episode of withdrawal. That is, strengthening of synapses occurred in the prelimbic cortex as a consequence of previous withdrawal episodes. This would lead one to speculate that if it had been possible to conduct electrophysiological recordings of neurones in the prelimbic cortex of repeatedly withdrawn rats, the capacity for long-term potentiation would have been increased due to the increased plasticity in the prelimbic cortex as indicated by the increased zif268 expression in the RWD animals. As previously discussed, this finding may partially support reported results by Borlikova and colleagues (Borlikova, Le Merrer et al. 2006) which found no difference in plasticity in limbic brain regions between the control group and the repeatedly withdrawn group. RWD group sis not induce higher levels of zif268 expression compared to SWD and CON rats except in PrL which suggests RWD leads to a “toughening up” of neurones exposed to previous ethanol withdrawal experience.

In conclusion, this present study showed that repeated ethanol withdrawal led to a general increase in neuronal activation of prefrontal cortical areas and neuronal plasticity was increased in the prelimbic cortex, which has been implicated in impulsive choice as well as fear conditioning. The general increase in neuronal activation of the prefrontal cortex provides an indication of the sensitivity of these brain areas to repeated ethanol withdrawal and furthermore, may predict a wide range of behaviours, mediated by the prefrontal cortex, including different varieties of impulsivity, decision-making tasks and attentional tasks, which may be expressed as a consequence of withdrawal.

Chapter 4

The effects of repeated ethanol withdrawal on two choice serial reaction time task performance.

4.1. Introduction:

Impulsivity may be defined as "actions that are poorly conceived, prematurely expressed, unduly risky, or inappropriate to the situation and that often result in undesirable outcomes" (Daruna 1993). Although impulsivity is a key diagnostic criterion in several psychiatric disorders, it is not explicitly defined in the Diagnostic and Statistical Manual (DSM IV). Impulsivity plays a role in normal as well as pathological behaviours, and has been implicated in substance abuse disorders for alcohol (Mitchell, Fields et al. 2005; Chen, Porjesz et al. 2007; Rubio, Jimenez et al. 2008; Lawrence, Luty et al. 2009; Lawrence, Luty et al. 2009), cocaine (Dalley, Fryer et al. 2007; Belin, Mar et al. 2008; Economidou, Pelloux et al. 2009; Winstanley, Bachtell et al. 2009) and nicotine (Spillane, Smith et al.; Doran, Spring et al. 2007; Bickel, Yi et al. 2008; Diergaarde, Pattij et al. 2008), as well as other psychopathologies such as attention-deficit hyperactivity disorder (Rubia, Cubillo et al.; Fox, Hand et al. 2008; Connor, Chartier et al. 2010; Dowson and Blackwell 2010). Interestingly, impulsivity has been found to correlate with both the early onset of drug use and later drug abuse (Disney 1999). There is much debate over the concept of impulsivity and the role it contributes to various psychopathologies; possibly due to the numerous varieties of impulsivity. Impulsivity is a multifaceted construct encompassing both personality traits of sensation seeking, novelty seeking, behavioural deficits in response inhibition and behavioural switching, cognitive impulsivity (also known as impulsive choice), motor impulsivity and premature responding. The reason for such diverse definitions of impulsivity lie in the lack of consensus amongst investigators on its definition and appropriate measures (Evenden 1999).

4.1.1. Impulsivity and the PFC:

The relationship between impulsivity and the prefrontal cortex is complex. Although impulsivity may be defined as a deficit in response inhibition, i.e. an individual

responding inappropriately or prematurely (motor impulsivity), it is also defined as a preference to choosing smaller, immediate rewards over larger delayed rewards (also referred to as cognitive impulsivity). This may be true of a drug addict, who will persistently choose the euphoric "high" of the drug experience over natural or long-term rewards. Several studies have found that individuals with prefrontal cortical damage exhibit similar cognitive deficits as drug addicts (Goldstein, Volkow et al. 2001; Manes, Sahakian et al. 2002; Bolla, Eldreth et al. 2003; Clark, Manes et al. 2003; Bechara 2004; Ersche, Fletcher et al. 2005; Koenigs and Tranel 2007). Impaired cognitive functions have an important role in perpetuating drug abuse and predisposing drug users towards relapse. For example, the compromised ability of cocaine users to inhibit prepotent responses has been associated with reduced activity in the anterior cingulate gyrus and the prefrontal cortex (Hester and Garavan 2004), evidence that is consistent with deficits found in alcohol and cocaine addicts, assessed using traditional neuropsychological measures (Goldstein, Leskovjan et al. 2004). The striatum has been implicated in motor impulsivity, a key brain area involved in drug addiction (Jentsch and Taylor 1999; Dalley, Fryer et al. 2007; Dalley, Mar et al. 2008; Carmona, Proal et al. 2009). However, the striatum is densely connected with the prefrontal cortex, particularly between the medial PFC and dorsal striatum (Alexander, DeLong et al. 1986; Groenewegen and Berendse 1994), there is a substantial likelihood that damage to the striatum may result in altered PFC function. The hypothesis proposed by Jentsch and Taylor (Jentsch and Taylor 1999) suggests that long term exposure to drugs of abuse change the function of cortical and subcortical brain regions, in which brain circuits involved in motivation, learning, memory and cognition are disrupted. This disruption may lead to a reduced capability for response inhibition, due to impaired frontal cortical function, and increased control of behaviour by sub-cortical limbic systems, which may result in behaviours centred towards reward-related stimuli. Jentsch and Taylor suggest that chronic exposure to drugs may lead to profound impulsivity which may contribute to the compulsive drug-seeking and drug-taking behaviour, which is the definitive hallmark of addictive behaviour.

Additionally, changes in PFC structure and function occurring as a direct result of drug abuse (Volkow, Fowler et al. 2003) and / or withdrawal (Duka, Gentry et al. 2004) may

result in behavioural inflexibility, observed in behaviours typified by an inability to withhold a prepotent response and insensitivity to changes in outcome value (devaluation). In an attempt to understand the behavioural processes and underlying brain mechanisms involved in addiction and relapse, Volkow and associates identified four neural circuits involved in addiction (Volkow, Fowler et al. 2003), which include circuits involved in reward (encompassing the nucleus accumbens and amygdala), motivation (e.g. caudate nucleus), learning and memory (amygdala and hippocampus), and cognitive and behavioural flexibility (e.g. the orbito-frontal cortex and anterior cingulate cortex). Memories associated with drug taking are proposed to be activated by environmental cues or contexts which generate positive expectancies about the drug which subsequently activates an individual's internal motivational state via the four aforementioned neural circuits. The behavioural choice to choose to take the drug is mediated by the prefrontal cortical areas involved in inhibitory control, which is involved in this decision making process. Continued and progressive drug taking weakens prefrontal cortical function regulation which in turn weakens the motivation, memory and reward circuits, subsequently leading to increased subcortical control of behaviour resulting in drug escalation which becomes compulsive (Volkow, Fowler et al. 2003).

4.1.2. Impulsivity in humans:

Impulsivity in human studies refer to a number of different behaviours including rash actions arising from "sensation seeking, risk- taking, novelty seeking, boldness, adverturesomeness, boredom susceptibility, unreliability and unorderliness" (Depue and Collins 1999). Whiteside and Lynam have attempted to map these concepts into behavioural traits (Whiteside and Lynam 2003), which include positive and negative urgency (relating to rash actions when experiencing positive and negative mood respectively), lack of planning (acting without forethought), lack of perseverance (referring to a failure to tolerate boredom), and sensation seeking (referring to the tendency to seek thrilling stimulation). The difficulty with translating human impulsivity into animal models lies in the issue that human impulsivity traits are derived from personality inventories; hence, one must consider how trait impulsivity

manifests itself in behavioural terms which can then be adapted into homologous animal models of impulsivity.

4.1.3. Impulsivity in animals:

There have been a number of behavioural paradigms used to investigate impulsivity in rodents. One of the most popular is the five choice serial reaction time task (5-CSRTT) devised by Carli et al (Carli, Robbins et al. 1983) originally as an analogue of the continuous performance test in humans and is used to assess visuospatial attention and motor impulsivity. In the 5-CSRTT, the subject is presented with a large number of discrete consecutive trials, in which the subject is required to wait during a fixed or variable inter-trial interval (ITI) whilst scanning the horizontal array of nose-poke apertures. Subjects are required to nose-poke into the aperture in the spatial location of a brief light stimulus in order to gain a food reward. A nose-poke response made during the ITI, prior to the presentation of the brief light stimulus is considered a premature response. A higher number of premature responses are thought to reflect higher levels of impulsivity (Muir, Everitt et al. 1996; Harrison, Everitt et al. 1997). Omissions may reflect motivational, sedative or motor effects and can be a good indicator for attentional impairments. The 5-CSRTT is generally favoured as it provides several independent measures of attention and recruits frontal neural systems implicated in impulsivity disorders (Robbins 2002; Dalley, Cardinal et al. 2004). Other methods of measuring motor impulsivity include the differential reinforcement of low rate of responding (DRL), the go / no go task and the stop signal task. On the DRL schedule of reinforcement, rats are required to space their responses by a specified time interval in order to obtain food reward. The go / no go task and the stop signal task both test a subjects' ability to inhibit a pre-potent response.

Impulsive choice relates to decision making and is measured using a delay discounting paradigm, in which impulsive behaviour is defined by a greater tendency to value and choose smaller, immediate rewards over larger, delayed rewards, despite being economically advantageous to choose the latter (Evenden 1999; Cardinal, Pennicott et al. 2001).

The PFC is well known to play a crucial role on inhibitory control of behaviour. For instance, selective lesions to the rat medial PFC lead to impairments in simple measures of behavioural inhibition (Jaskiw, Karoum et al. 1990; Dalley, Thomas et al. 1999). Impulsivity on the 5-CSRTT is generally increased by lesions to the PFC, especially to the anterior cingulate cortex (Muir, Everitt et al. 1996), the infralimbic cortex (Chudasama, Passetti et al. 2003) and lesions that disconnect the medial PFC from the anterior medial striatum (Christakou, Robbins et al. 2001) hence providing support for the role of the PFC in motor impulsivity via its high connectivity with the striatum.

4.1.4. Effects of chronic alcohol and repeated ethanol withdrawal on PFC:

Heavy alcohol consumption causes neurodegeneration that contributes to impaired executive functions. Human alcoholics have been found to have lower brain volumes of cortical and subcortical brain structures (Crews and Nixon 2009). Progressive increases in ethanol consumption lead to changes in brain structure which reduces behavioural control and promotes further alcohol abuse. Heavy alcohol use can result in impulsive behaviour (Jentsch and Taylor 1999; Goldstein and Volkow 2002). There is a progressive effect of alcohol on impulsivity as increasing levels of dependence can result in decreasing levels of self-control (Koob and Le Moal 1997). There is also an indication that impulsivity can predict the development of alcohol abuse (Dawes, Tarter et al. 1997). As it is unclear whether impulsivity increases the risk for development of alcohol use disorders or whether it arises from heavy alcohol consumption which predisposes a person to alcohol abuse, it would be important to investigate this further in animal models.

The hypothesis on which the current set of experiments are based, is that repeated episodes of ethanol withdrawal increase the severity of subsequent withdrawal symptoms. This hypothesis has been supported by several clinical studies showing a positive relationship between the number of withdrawals and increased seizure susceptibility in alcoholics (Ballenger and Post 1978; Brown, Anton et al. 1988; Booth and Blow 1993). Alcoholic patients have been reported to perceive more fear in all facial expressions compared with social drinkers (Townshend and Duka 2003) and this exaggerated fear perception was positively associated with the number of alcohol

detoxifications which suggests the amygdala, the brain area involved in fear processing, undergoes kindling-like processes which result in increased withdrawal severity. There are hints of increased impulsivity as measured by the inability to withhold a prepotent response in female bingers, however no significant differences in this measure of impulsivity were found in male bingers (Townshend and Duka 2005). In animal studies, rats that had undergone repeated episodes over-responded on a fixed interval paradigm, indicating an inability to wait for the appropriate time to respond (Borlikova, Elbers et al. 2006), which is suggestive of changes in prefrontal cortical function. Additionally, repeatedly withdrawn rats were slower in learning to suppress non-rewarded responses in a negative patterning task (Borlikova, Elbers et al. 2006) Although repeatedly withdrawn rats were not slower at task acquisition of a conditioned response for a single-element stimulus in the negative patterning task, they appeared to show a deficit in learning about compound stimuli compared with singly withdrawn rats. There have been indications in previous experiments from this laboratory that ethanol withdrawal may give rise to impulsive responding. In an operant experiment of conditioned emotional fear, repeated ethanol withdrawal impaired a rat's ability to suppress responding and impaired extinction behaviour (Ripley, O'Shea et al. 2003). Extinction involves inhibitory learning hence it is possible that this form of behaviour recruits the capacities of the prefrontal cortex, thus providing a plausible interpretation for the effects of repeated ethanol withdrawal on impaired extinction behaviour.

4.1.5. The Two-Choice Serial Reaction time task (2-CSRTT):

To our knowledge, the effects of repeated ethanol withdrawal on impulsivity using the 5-CSRTT have not yet been examined. In an attempt to address this issue, we have devised an analogous two choice serial reaction time task (2-CSRTT). In this present study, the 2-CSRTT paradigm was devised to test rats' attentional capacity and impulsive behaviour as a result of repeated ethanol withdrawal. The 2-CSRTT paradigm was modelled on the 5-CSRTT, using the same measures of attention and impulsivity, and adapted to be conducted using the two lever operant equipment as employed in previous studies conducted in this laboratory in order to maintain consistency with previous studies.

Within a daily session, lasting 60 minutes, each animal subject was presented with a large number of discrete trials. A trial was automatically initiated by MedPC programme, and signalled by the illumination of the house light. The subject was required to wait a variable or fixed delay time before the presentation of one of the cue lights (left or right cue light) in order to make a lever press response in the spatial location of the cue light stimulus (left or right lever) to receive a food reinforcement (sweetened food pellet).

Measures of correct responding on the 2-CSRTT were similar to the 5-CSRTT, where stimulus accuracy represents correct responding as a percentage of all discrete trials (excluding premature responses and omissions). Premature responses were determined by the number of responses made prior to stimulus presentation and omissions were recorded if the rat failed to respond during the imposed delay time (set at 0,2,5 and 10 seconds).

The rat model of repeated ethanol withdrawal in this study has been well established in this laboratory. This paradigm of forced ethanol consumption used the chronic administration of 7% ethanol-containing liquid diet (Dyets, USA) as a sole food source. Rats experienced either single or repeated ethanol withdrawal (the 7% ethanol containing liquid diet was identical). Single ethanol withdrawal involved 24 consecutive days of ethanol liquid diet administration with a final withdrawal at the end of treatment. Repeated ethanol withdrawal comprised 2 x 3-day intermediate withdrawal episodes and a final withdrawal at the end of ethanol treatment. Repeated ethanol withdrawal treatment lasts 30 days in total, with 24 days of total ethanol exposure, equivalent to that of single ethanol withdrawal.

4.1.6. Experimental Aims:

The aim of this present study was to assess the effects of single and repeated ethanol withdrawal on performance measures in a 2-CSRTT paradigm for evidence of attentional deficits and impulsive behaviours.

4.2. Materials and Methods:

4.2.1. Experiment 1: Characterisation of 2-CSRTT.

In order to investigate the baseline responding of rats in the novel 2-CSRTT, rats were presented with a large number of discrete consecutive trials in which subjects were required to wait during a variable delay time (0,2,5 or 10 seconds), and to lever press on one of 2 levers at the spatial location of a brief visual stimulus (cue light flash lasting 1 second) to earn a food reinforcement. Lever press responses made prior to the cue light presentation were recorded as premature responses. Increases in premature responses are thought to reflect increased impulsivity (Muir, Everitt et al. 1996; Harrison, Everitt et al. 1997). As this 2-CSRTT paradigm was modelled on the 5-CSRTT, it shares the advantage of providing similar independent measures of attentional performance including visual discrimination and response inhibition (Robbins 2002). As successful performance on the 5-CSRTT has been found to recruit the PFC, the OFC and striatum (Robbins 2002; Dalley, Cardinal et al. 2004), it is reasonable to assume that successful performance on the 2-CSRTT involve activation of the same brain areas.

This experiment was conducted to assess the behaviour of the control group in the 2-choice serial reaction time task. Rats were ats were fed a nutritionally complete control liquid (Dyets, USA) the basis for future experiments in which the diet served as a carrier for ethanol. This experiment investigated control animals' baseline performance on 2-CSRTT performance using variable delay times (0, 2, 5, 10 seconds).

4.2.2. Subjects:

Eight male Lister hooded rats (Charles River, UK) weighing 175 – 195g at the start of the experiment were used. Acclimatisation and food restriction conditions were identical to those detailed previously in chapter 2, section 2.2.2.

4.2.3. Control Liquid diet treatment:

Control liquid diet treatment was identical to the procedure detailed in chapter 2, section 2.2.1. Control liquid diet was administered after rats had reached criterion on the 2-CSRTT (criterion consisted of >70% discrimination accuracy for 3 consecutive sessions, with a light stimulus duration of 1 sec.)

4.2.4. Apparatus:

The test apparatus consisted of 8 standard two-lever operant conditioning chambers (Med-Associates) with a steel rod floor. The response wall consisted of a central food magazine (ENV-203M, Med-Associates) connected to a pellet dispenser. A single 45mg food pellet (Test Diet (AIN-76A) Sandown Scientific, Middlesex, UK) was delivered for each correct response. Located at the entrance of the food magazine was an infra-red photocell beam which monitored the latency in which food was collected at each trial, and two lever response units (Med-Associates), located beneath two cue lights (ENV-221M, Med-Associates) on either side of the central response panel.

The operant chambers were individually housed within sound attenuating cabinets and were ventilated by low-level noise fans which also served to block out background noise. The apparatus was controlled and data was collected using an IBM-compatible PC with a Windows 2000 operating system running MedPC version IV (Med-Associates).

4.2.5. Behavioural Training:

1. **Magazine training:** In this phase of training, rats learned to associate food reward with the onset of the cue lights and the food magazine. Rats were trained to collect food pellets from the magazine delivered at 1-minute intervals at the same time as the onset of both cue lights (which were illuminated for 5 seconds). When rats reached criterion, they progressed onto the next stage if, in their final 20 trials of the session, they achieved greater than 15 correct responses, where correct responses were head entries into food magazine while magazine light was on (duration 10 seconds).
2. **Lever pressing:** Rats were trained to lever press and associate the lever press with a cue light and food delivery. Two levers were presented throughout the whole 60-minute session and the house light was illuminated throughout the entire session. Both levers were active on FR1 schedule. A response on either lever resulted in delivery of a food pellet and the cue light over the pressed lever briefly flashed for 0.5 second. Rats were trained in 60 minute daily sessions until they reached criterion, which consisted of the the rat more than 30 reinforcers, and lever presses on both sides to be greater than 30% and less than 70% of the total

number of responses, and the correct number of nose pokes into the food magazine must be equal to or greater than 15.

3. Behavioural Procedure: Discrete trials

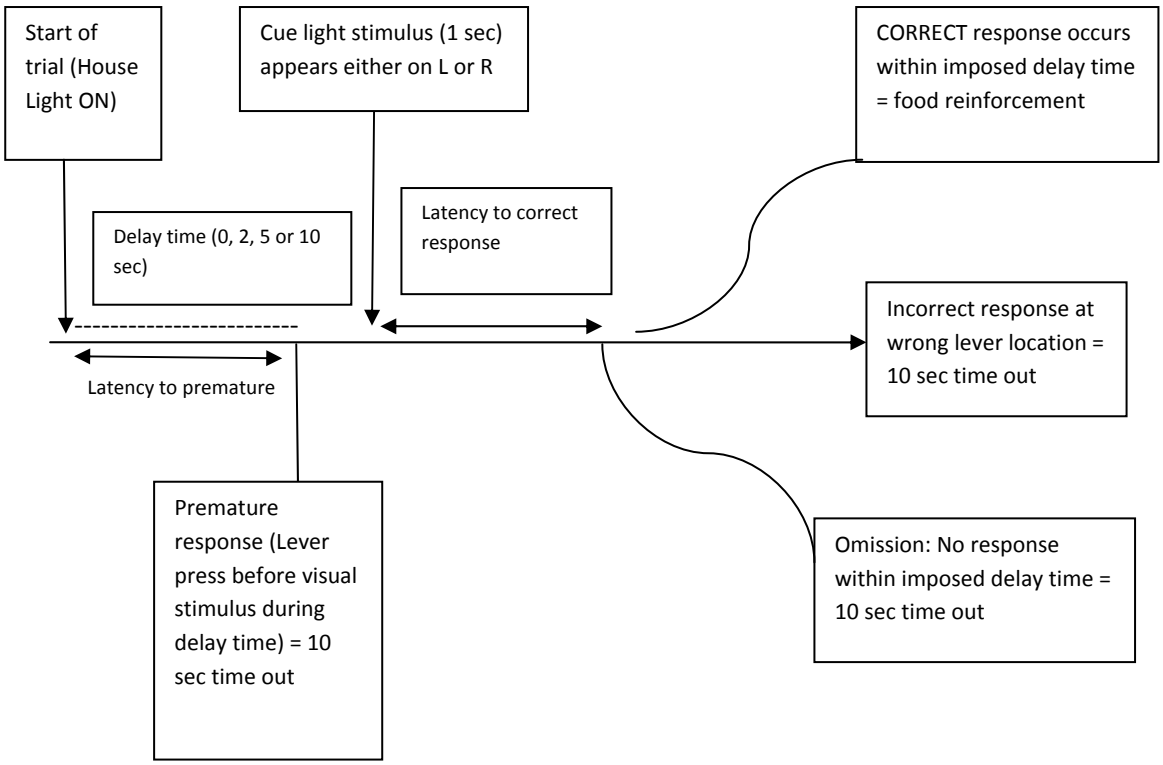


Figure 4.1 Diagram showing discrete trials in the 2-CSRTT. This diagram details 1 discrete trial only. A one hour daily session consisted of approximately 114 discrete trials.

Rats were trained to respond to a brief visual stimulus (illumination a cue light for 1 second, presentation was randomly selected between left and right for each trial). At the beginning of each session, the house light was illuminated one minute after the program started, which allowed rats to acclimatise to the apparatus. The onset of one of the two cue lights was presented after a delay time of 0, 2, 5 or 10 seconds. The delay time was selected by random generation, to present approximately 20 – 25 trials of each delay time, hence within a 60-minute session, a rat was exposed to approximately 114 trials. Levers were active on an FR1 schedule, only for correct responses. The delay time in this experiment was analogous to the inter trial interval (ITI) used in the 5 choice serial reaction time task.

Responses on the lever under the illuminated cue light were recorded as correct responses, and were rewarded with a food pellet delivered into the central magazine, after which the subject could collect and consume the pellet. The magazine light was illuminated at the point of pellet delivery to focus the subject's attention on the food reinforcement. Eight seconds after a correct lever press, the house light was extinguished for 10 seconds prior to the start of the next discrete trial.

Responses on the incorrect lever (i.e. the lever under the unilluminated cue light during the cue light presentation) werewere recorded as incorrect responses and led to a 10-second time out period (i.e. the house light was extinguished for 10 seconds) before the start of the next trial. Failure to respond within the duration of the cue light illumination were recorded as an omission and led to punishment by a 10-second time out period.

A premature response was recorded when a rat made a press response prior to the cue light onset. This led to a 10-second time out period before the start of the next discrete trial.

During any one session, the cue light presentation was presented an equal number of times from left and right cue lights, and the four different delay times were also presented approximately equally (so in every 100 light stimulus presentations, each delay time was presented approximately 25 times). A daily session consisted of approximately 114 trials and lasted 60 minutes. For the first session of training, the presented cue light duration was 5 seconds and, once criterion was reached, was reduced to 2 seconds, then 1 second. Rats reached criterion when they attained equal to or greater than 70% correct responses for 3 consecutive daily sessions. The mean number of sessions required to reach criterion on all stages of training was 32.

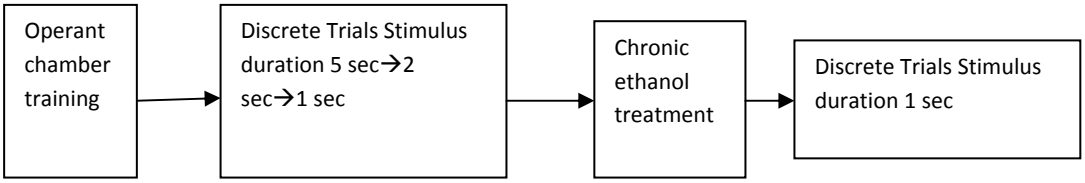


Figure 4.2 Diagram showing progression of experiments 1 and 2.

4.2.6. Performance measures:

% correct responses: number of correct responses taken as a percentage of correct responses, incorrect responses and omissions.

% omissions: number of omissions taken as a percentage of correct responses, incorrect responses and omissions.

% Premature responses: Premature responses were calculated as a percentage of all discrete trials (including premature responses, correct responses, incorrect responses and omissions).

4.2.7. Data analysis:

Data were analysed by ANOVAs using SPSS Version 16. Graphs were plotted using GraphPad Prism 4.0. All tests of significance were performed at $\alpha = 0.05$. All tests with $\alpha < 0.1$ were regarded as a trend. Homogeneity of variance was verified using Levene's test. For repeated measures analyses, Mauchley's test of sphericity was applied and the degrees of freedom were corrected using Greenhouse Geisser ε values when assumptions of sphericity were violated. Significant main effects were further analysed using pair wise comparisons with a Bonferroni correction.

In the 2-CSRTT, 3 variables were analysed in 3 separate ANOVAs: % premature responses, % correct responses and % omissions.

4.3. Results: Experiment 1: Characterisation of the 2-CSRTT.

4.3.1. Overview

During the first 3 sessions, correct responding was at the highest level at 2 and 5 second delay times, whereas at 0 and 10 second delay times, correct responding decreased. However, the decreased correct responding at 0-second delay time was primarily due to the high percentage of omissions, which suggested that at 0-second, rats did not detect the light stimulus therefore attentional capacity was not sufficiently high to respond to the shortest delay time. As the cue presentation at 0-second delay time was simultaneous with the onset of the house light cue, it was possibly the least salient presentation of all the delay times, which may account for the lack of response at 0-second delay. It was also highly unlikely that an animal would be able to respond prematurely at 0-second delay time.

The decreased correct responding at 10-second delay time was attributed to greater percentage of premature responding, which indicated that under baseline conditions in the absence of alcohol, rats were initially unable to withhold premature responding.

Upon reaching criterion, correct responding at 0-second delay did not improve, indicating that responding at the shortest delay time required a level of attentional capacity which was too stringent. Nonetheless, at 2, 5 and 10-second delays, correct responding increased. Correct responding at 10 seconds was lower than at 2 and 5 seconds, although this result was confounded by increased opportunity to make a premature response, indicating that with extended training, premature responding at 10 second delay was marginally increased compared with premature responding at 2 and 5 second delay times, and rats were unable to withhold premature responding. There was also a possibility that 10-second delay time was too long for the subjects to resist premature responding.

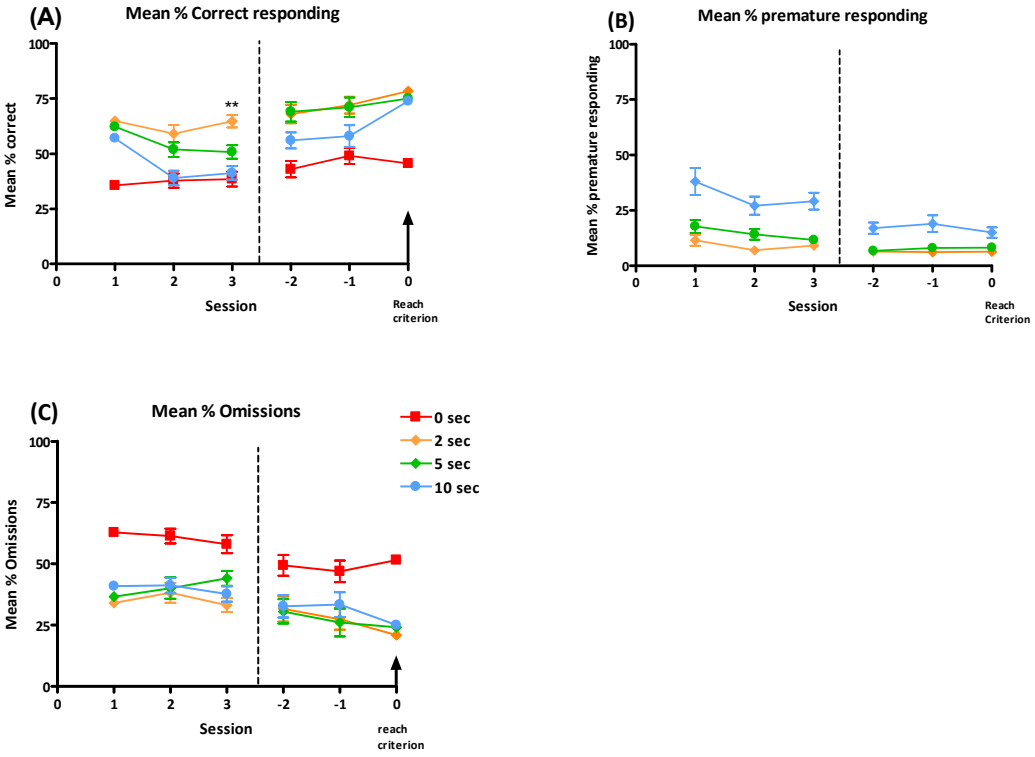


Fig 4.3: Mean±sem performance on 2-CSRTT on the first 3 days of testing (Days 1 - 3) and the last 3 days on reaching criteria (Days -2 to 0) with 1 sec stimulus duration. Data show responses for delay times of 0, 2, 5 and 10 seconds. (A) Mean % correct responding (B) Mean % premature responding and (C) Mean % omissions.

4.3.2. Baseline Correct responding:

Analysis of baseline levels of mean % correct responding on the first 3 days of 2-CSRTT testing (**see figure 4.3A**) show the highest levels of correct responding in each session occurs at 2 second and 5 second delay. Lowest levels of mean % correct responding occurred at 0 sec delay.

There was a significant main effect of delay, ($F_{(3, 63)} = 34.3$, $p < 0.05$). Post hoc comparisons show correct responding at 2 sec delay was significantly higher than correct responding at 5 sec delay ($p < 0.001$) on day 3, indicating correct responding improved with the number of sessions and 2-second delay was the optimal performance time delay. No significant difference was found in correct responding between 0 sec delay and 10 sec delay ($p = 1.00$), however, correct responding at 5 sec ($p = 0.001$) and 10 sec ($p < 0.001$) delay was significantly higher compared to correct responding at 0 sec delay.

On the final 3 days, during reaching criterion, there was significant main effect of delay ($F_{(3, 57)} = 18.8$, $p < 0.001$) which showed the highest level of correct responding again occurred at 2 second delay time ($p < 0.001$). The lowest level of correct responding occurred at 0 sec delay. There was no significant difference between the correct responses made at 2 sec and 5 sec delay time ($p = 1$) which indicated that between 2 and 5 second delay times, there was little effect on optimal performance in the 2-CSRTT.

4.3.3. Premature Responses:

As illustrated by **Figure 4.3B** showed premature responding during the first 3 sessions increased with delay time ($F_{(2,26)} = 4.2$, $p = 0.04$). Premature responding at 10 sec delay time was higher than at 2 or 5 sec delay time ($p = 0.09$) although this finding was not statistically significant. In the last 3 days upon reaching criterion, premature responding at 10 sec delay time was found to be significantly higher than at 2 or 5 sec delay times. ($F_{(2,18)} = 12.7$, $p = 0.005$) indicating that with practice, although premature responding was reduced, the longer delay time of 10 seconds did not reduce premature responding to the equivalent level of 2 or 5 second delay times.

4.3.4. Conclusions:

This experiment was conducted to test the experimental parameters of the 2-CSRTT and to measure the baseline performance of rats on the 2 choice serial time task paradigm prior to receiving chronic ethanol liquid diet. The 2-CSRTT was a novel behavioural paradigm which included behavioural measures also used in the 5-CSRTT.

The highest levels of correct responses occurred at the 2-second delay time which was significantly higher than correct responses at 0, 5 or 10 second delay times, indicating that the 2 second delay was one in which rats could demonstrate optimal performance on the 2-CSRTT. The lowest levels of correct responding occurred at 0 sec delay time. The low level of correct responding at 0-second delay time with the concomitant high percentage of omissions suggested the 0-second delay as an experimental condition was too stringent for the animal subjects to respond correctly. The decreased level of correct responding at 10 second delay time, accompanied by high levels of premature responding in comparison to the other delay times, indicated

that the longer that subjects were required to wait for a cue light stimulus, the higher the levels of premature responding. Measures of correct responding, premature responding and omissions all improved with extended training.

4.4. Experiment 2: Consequences of repeated ethanol withdrawal on 2-CSRTT performance.

Experiment 2 investigated the effects of repeated ethanol withdrawal on the attentional capacity and impulsive behaviour of rats. Subjects were trained to stable performance on the 2-CSRTT up to stimulus duration of 1 sec prior to chronic ethanol treatment administration. Performance on the 2-CSRTT was tested 2 weeks after the final withdrawal.

4.4.1. Subjects:

Twenty four four male Lister hooded rats (Charles River, UK) weighing 175 – 195g at the start of the experiment were used as experimental subjects. Acclimatisation and food restriction conditions were identical to those detailed previously in chapter 2, section 2.2.2.

4.4.2. Chronic Ethanol Liquid diet treatment:

Ethanol liquid diet treatment was identical to the procedure detailed in chapter 2, section 2.2.1. Ethanol liquid diet was administered after rats had reached criterion on the 2-CSRTT (criterion consisted of >70% correct responding for 3 consecutive sessions, using a light stimulus duration of 1 sec.

4. 4.3 Results: Ethanol consumption:

There was no significant difference in the ethanol consumption between SWD and RWD groups over the final 7 days of chronic ethanol treatment. Rats consumed a mean amount of 21-22 g ethanol / kg of body weight / day.

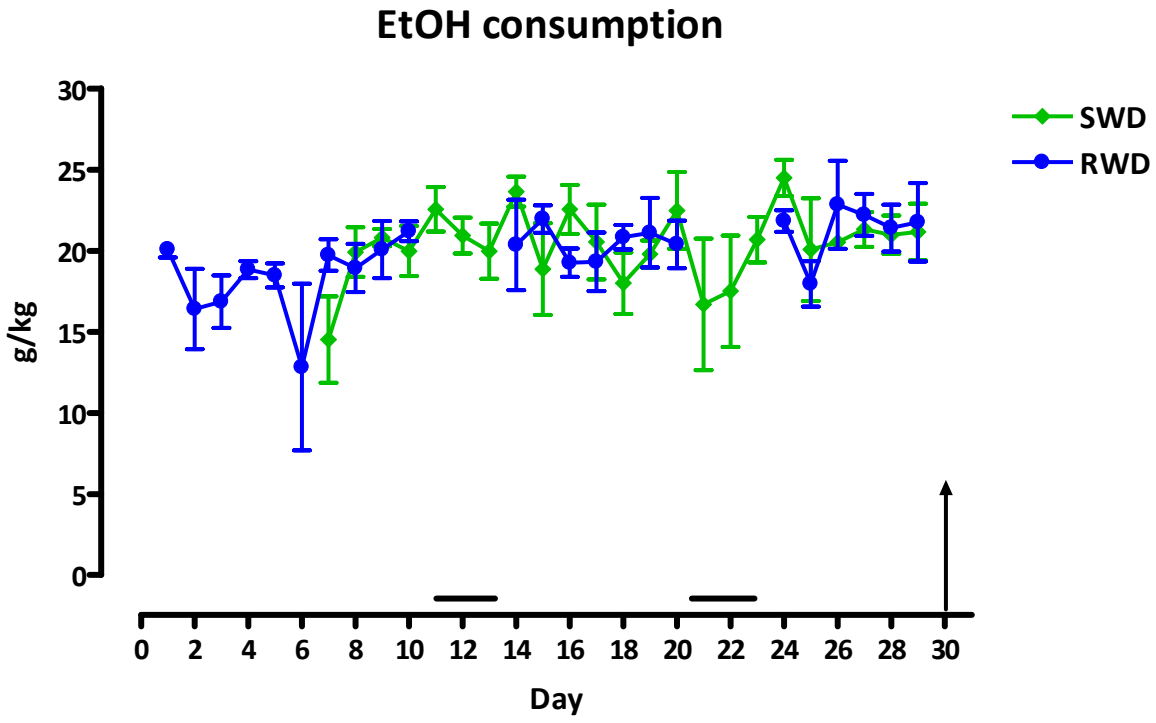


Fig 4.4: Mean± SEM ethanol consumption: Ethanol consumption expressed as g per kg of body weight per day of treatment. Arrow indicated the final withdrawal; intermediate withdrawal episodes in the RWD group took place on treatment days 11-13 and on treatment days 21 – 23.

Statistical analysis using a repeated measures ANOVA found no significant group difference in ethanol consumption between the SWD and RWD groups ($F_{(1,6)} = 0.32$. $p = 0.59$) (as illustrated by **fig 4.4.**) In the last 7 days of treatment, RWD group consumed a mean of 21.3 g/kg of ethanol, the SWD group consumed a mean amount of 21.6 g/kg of ethanol.

4.4.4 Two choice serial reaction time task performance: Trials to criterion

As demonstrated in **figure 4.5**, there were no differences in the number of trials to criterion in the 2-CSRTT between the 3 treatment groups.

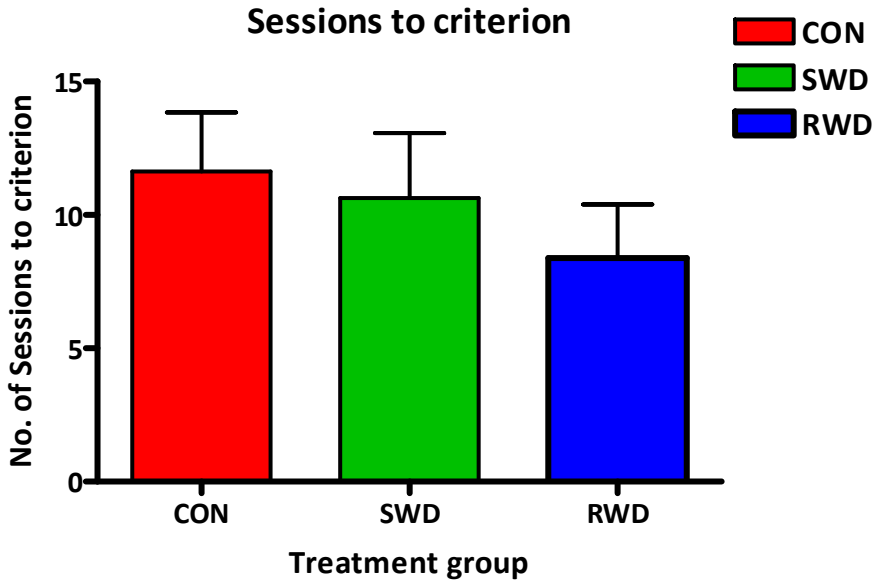


Fig 4.5: Number of sessions to criterion after the final withdrawal for each treatment group.

Statistical analysis of the number of trials to criterion found no significant group differences ($F_{(2, 21)} = 0.64$, $p = 0.54$) indicating that chronic ethanol withdrawal did not significantly affect the number of sessions required to reach criterion in the 2-CSRTT.

4.4.5 Correct responses:

Correct responding at 0 and 10 second delay time was significantly lower compared with correct responding at 2 and 5 second delay times during the initial stages of alcohol withdrawal (Days 1-3). Correct responding increased with extended training (days -2 to day 0) although no significant differences were observed between the treatment groups, suggesting that chronic ethanol treatment and withdrawal did not significantly impair correct responding in the 2-CSRTT.

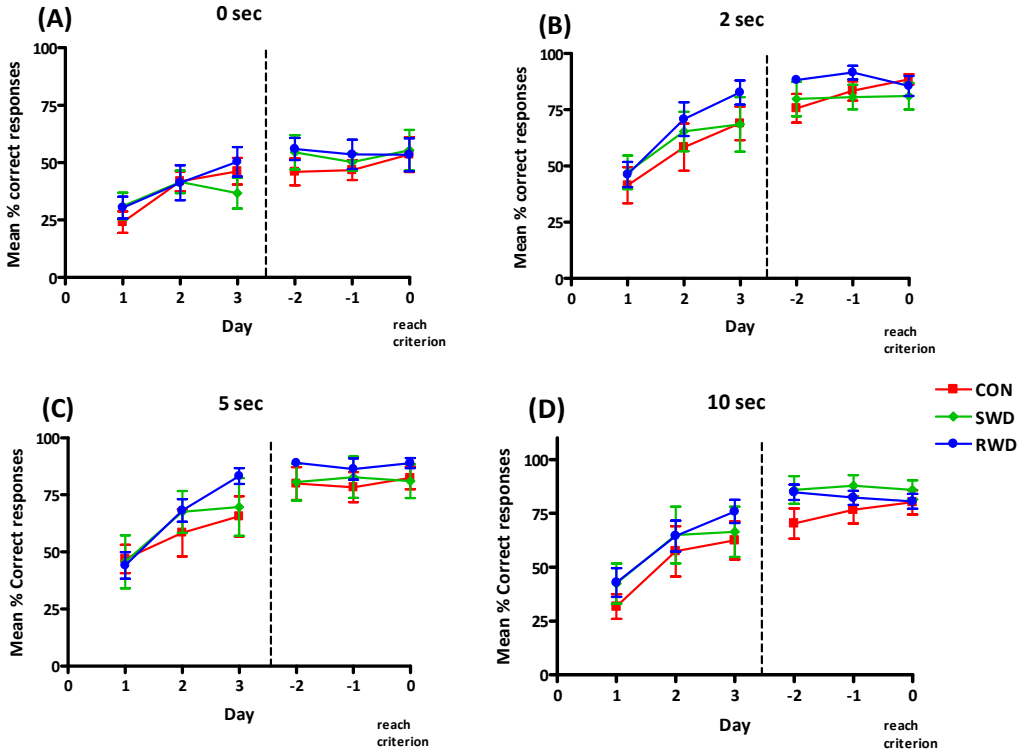


Fig 4.6: Mean ± SEM Correct responses in 2-CSRTT: Performance measured on the first 3 days of testing (Days 1 - 3) after the final withdrawal from ethanol and the last 3 days on reaching criteria (Days -2 - 0) post-withdrawal from ethanol.

As depicted by **Figure 4.6**, correct responding significantly increased with session number at all the time delays, for example at 0-sec delay ($F_{(5, 105)} = 13.4$, $p < 0.001$), at 2-sec delay ($F_{(5, 105)} = 25.7$, $p < 0.001$), at 5-sec delay ($F_{(5, 105)} = 23.2$, $p < 0.001$) and at 10-sec delay ($F_{(5, 105)} = 27.8$, $p < 0.001$). There were no significant effects of single or repeated ethanol withdrawal on correct responding after the final withdrawal at 0 second delay time ($F_{(2, 21)} = 0.348$, $p = 0.71$), at 2 second delay time ($F_{(2, 21)} = 1.127$, $p = 0.343$), at 5 second delay time ($F_{(2, 21)} = 0.657$, $p = 0.53$) and 10 second delay ($F_{(2, 21)} = 1.12$, $p = 0.346$).

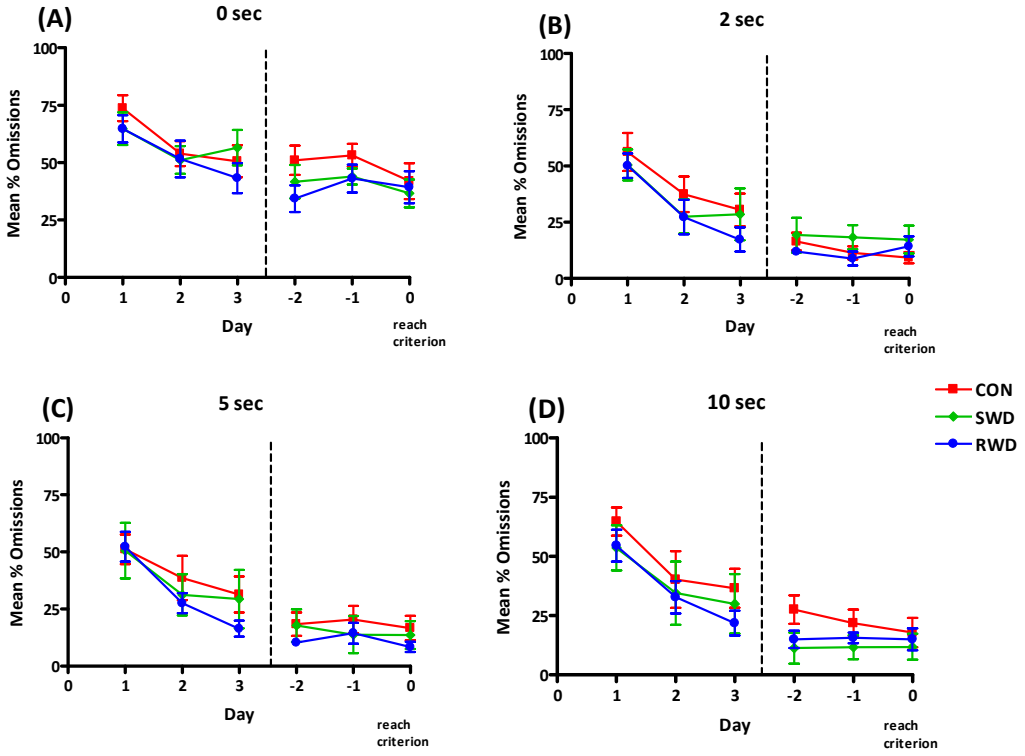


Fig 4.7: Mean % omissions in 2-CSRTT: Performance measured on the first 3 days of testing (Days 1 - 3) after the final withdrawal from ethanol and the last 3 days during reaching criteria (Days -2 - 0) post-withdrawal from ethanol. Graphs show omissions at (A) 0 sec ITI, (B) 2 sec ITI, (C) 5 sec ITI and (D) 10 secs.

4.4.6. Omissions:

There were no significant group x session interactions ($F_{(4, 42)} = 1.19$, $p = 0.329$). At each delay time, omissions decreased during the first 3 days post withdrawal, which may demonstrate a learning effect. For example at 0-sec delay ($F_{(2, 42)} = 15.4$, $p < 0.001$), at 2-sec delay ($F_{(2, 42)} = 18.87$, $p < 0.001$), at 5-sec delay ($F_{(2, 42)} = 13.57$, $p < 0.001$) and at 10-sec delay ($F_{(2, 42)} = 11.97$, $p < 0.001$). With extended training (upon reaching criterion of 70% correct responding for 3 consecutive days), the level of omissions remained stable. However, no significant differences between the groups were observed at 0-sec delay ($F_{(2, 21)} = 0.38$, $p < 0.69$), at 2-sec delay ($F_{(2, 21)} = 0.978$, $p = 0.393$), at 5-sec delay ($F_{(2, 21)} = 0.448$, $p = 0.645$) and at 10-sec delay ($F_{(2, 21)} = 0.77$, $p = 0.476$).

4.4.7. Premature responses:

Premature responses increased with delay time in the first 3 post-withdrawal days. However, with extended training, premature responses stabilised and remained below 25% at all delay times. No significant differences were found between the treatment groups.

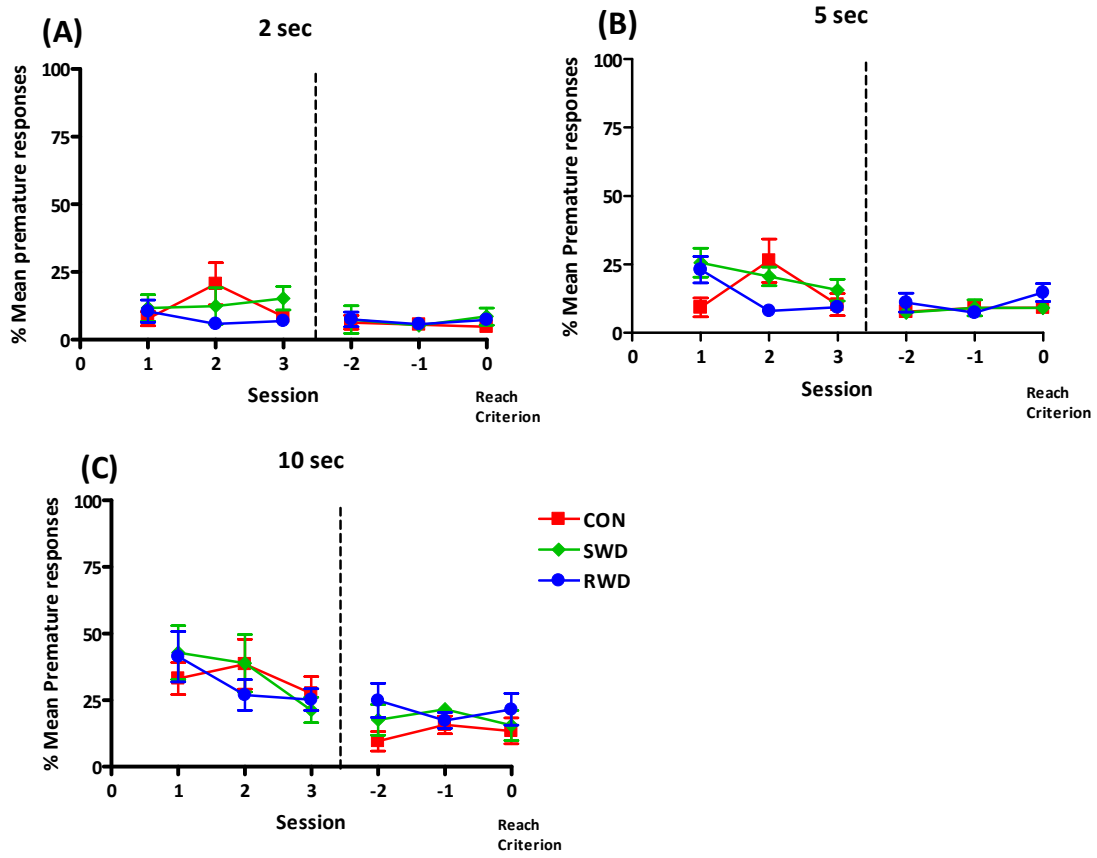


Fig 4.8: Mean±SEM % premature responses in 2-CSRTT: Performance was measured on the first 3 days of testing (Days 1 - 3) after the final withdrawal from ethanol and the last 3 days on reaching criteria (Days -2 - 0) post-withdrawal.

As illustrated by **Figure 4.8 (A, B and C),** no significant group effects observed in premature responses in the 1st 3 test sessions at 2 second delay time, ($F_{(2, 10)} = 2.15$, $p = 0.17$), 5 second delay ($F_{(2, 17)} = 2.12$, $p = 0.15$) and 10 second delay time ($F_{(2, 19)} = 0.025$, $p = 0.98$).

There was a trend for premature responses to decrease with session number ($F_{(2,16)} = 3.2$, $p = 0.067$) although this finding did not reach statistical significance . Premature

responses significantly increased with increasing delay times ($F_{(2,16)} = 33.7$, $p < 0.001$). It was not possible to conduct statistical analysis of the last 3 test sessions during attaining criterion as there were too few numerical values for premature responses.

4.4.8. Conclusions:

This current experiment found ethanol withdrawal did not significantly impair 2-CSRTT performance as no group differences in any of the measures were observed. There were no significant differences in the number of sessions to criterion, suggesting that single and repeated ethanol withdrawal did not affect rats' ability to acquire optimal performance on the 2-CSRTT.

There was a significant main effect of session number in all observed 2-CSRTT measures suggested a learning effect as performance improved over sessions. Premature responding decreased, correct responding increased and omissions decreased as the number of sessions progressed. Upon attaining criterion, subjects learned to withhold their premature responses, increased their attentional capacities (as observed in decreased omissions) and improved efficiency of responding by increased correct responding.

There were no group differences observed during early withdrawal. Findings from this current study indicated ethanol withdrawal did not affect performance on 2-CSRTT. It was possible the lack of effect of ethanol withdrawal observed in this present experiment may be attributed to lack of 2-CSRTT training during chronic ethanol treatment. After rats had undergone operant training and discrete trials during which they attained criterion, they were administered chronic ethanol treatment which lasted 24-30 days. For the duration of chronic ethanol treatment, no 2-CSRTT training was provided therefore as a result of 24-30 days break from 2-CSRTT training, rats may have lost their level of performance of pre-ethanol levels due to lack of practice.

4.5. Experiment 3: Effects of RWD on 2-CSRTT performance using variable delay times with chronic ethanol administration occurring prior to training.

In order to address the issues raised in experiment 2, of imposing a break in 2-CSRTT training, this experiment aimed to investigate the effects of repeated ethanol

withdrawal on 2-CSRTT performance after the administration of chronic ethanol liquid diet.

In experiment 3, we investigated the effects of single and repeated ethanol withdrawal on 2-CSRTT performance by training rats to criterion on the 2-CSRTT after administering chronic ethanol liquid diet. Rats were tested 2 weeks after the final withdrawal in protracted abstinence.

Training and testing on the 2-CSRTT was identical to experiments 1 and 2 with the exception of the time point at which ethanol liquid diet was administered. In experiment 3, rats were fed chronic ethanol liquid diet prior to training and testing on the 2-CSRTT occurred 2 weeks post-withdrawal. This experiment was conducted to investigate the effects of repeated ethanol withdrawal on 2-CSRTT performance when ethanol was administered prior to learning the task.

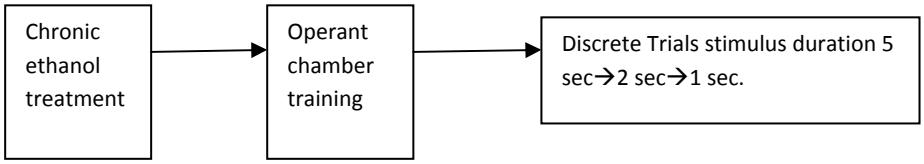


Figure 4.9: Progression of experiment 2

4.5.1. Subjects:

Twenty-four male Lister hooded rats (Harlan, UK) weighed 200-225g at the start of the experiment. Acclimatisation and housing conditions were identical to those detailed in chapter 2, section 2.2.2.

4.5.2. Chronic ethanol liquid diet:

Chronic ethanol liquid diet administration was conducted in a manner identical to that described in chapter 2, section 2.2.1, in which ethanol liquid diet treatment was administered prior to behavioural training. Rats were food deprived (20g/rat /day of standard rat chow) for a week prior to the start of behavioural testing.

4.5.3. Behavioural testing:

Training protocol and behavioural testing was identical to that previously described in the protocol of Experiments 1 and 2 (Section 4.2.5).

4.5.4 Results: Ethanol consumption

As illustrated by **Fig 4.10**, there was a significant difference in ethanol consumption between the groups on experimental days 17, ($F_{(1, 7)}$ = 13.41, p = 0.011), experimental day 18, ($F_{(1, 7)}$ = 8.796, p = 0.025) and experimental day 19 ($F_{(1, 7)}$ = 6.022. p = 0.05). On experimental days 17, 18 and 19, RWD group drank significantly more ethanol than SWD group. Statistical analysis was conducted on SWD and RWD group data for which the corresponding treatment days during which rats received ethanol liquid diet were analysed.

There were no significant group differences in ethanol consumption on any of the other experimental days (p > 0.05). In the last 7 days of treatment, RWD group consumed a mean amount of 18 g/kg of ethanol, whereas the SWD group consumed a mean amount of 17 g/kg of ethanol.

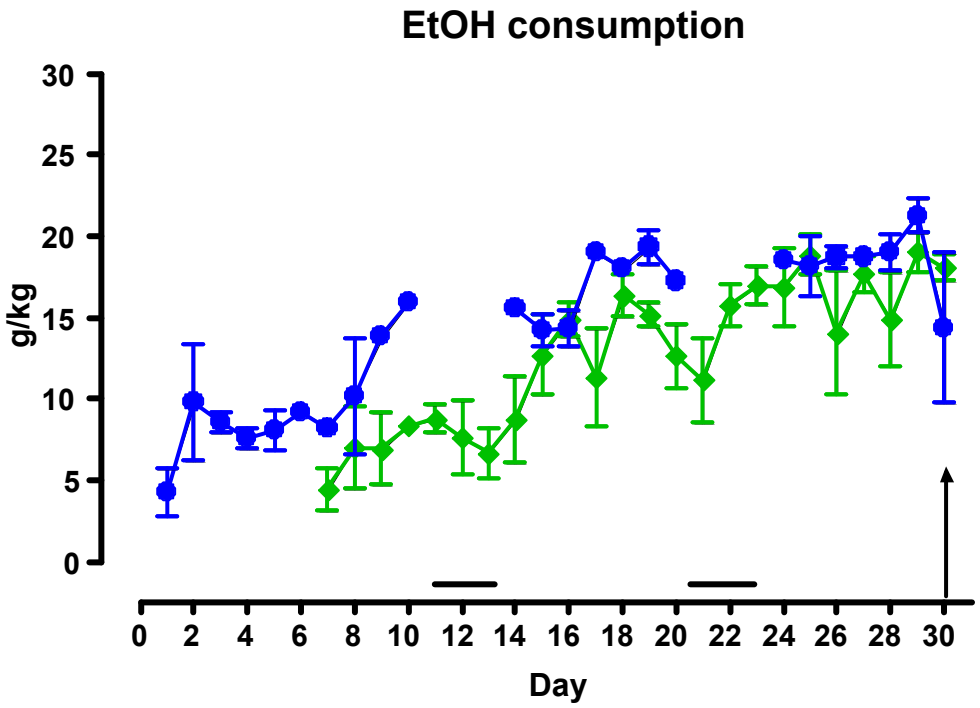


Fig 4.10: Mean ethanol consumption: Ethanol consumption was expressed as g per kg of body weight per day of treatment. Data represented the mean ± SEM. Arrow indicated the final withdrawal. During the last week of treatment, SWD and RWD rats consumed an average of 17 – 18 g/kg/day of ethanol.

4.5.5. Number of sessions to criteria in the two choice serial reaction time task.

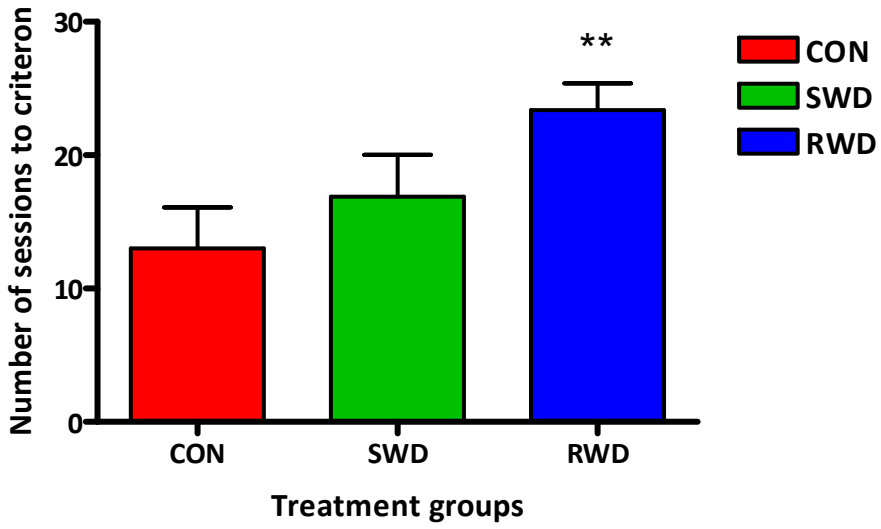


Fig 4.11: Number of sessions to criterion after the final withdrawal from ethanol. Criterion = 70% correct responses.

Statistical analysis of the number of trials to criterion using a one-way ANOVA found a significant main effect of group ($F_{(2, 21)} = 5.377$. $p = 0.013$). Post hoc tests revealed that RWD group required significantly more trials to reach criterion than CON ($p = 0.004$) and SWD ($p = 0.05$) rats, indicating repeated ethanol withdrawal impaired rats' ability to attain criterion on 2-CSRTT, which suggested a possible impairment of learning effect.

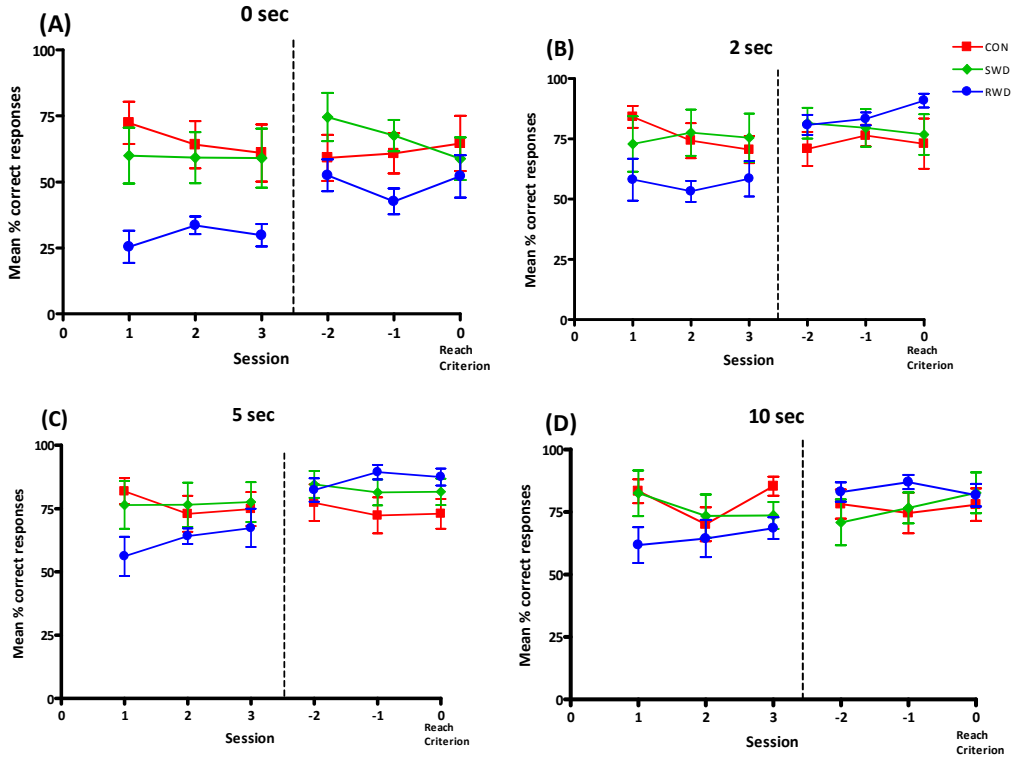


Fig 4.12: Mean % Correct responses in 2-CSRTT where training took place after chronic ethanol treatment was administered : Performance measured on the first 3 days of testing (Days 1 - 3) after the final withdrawal from ethanol and the last 3 days on reaching criteria (Days -2 - 0) post-withdrawal from ethanol.

4.5.6 Correct responding:

Figure 4.12 A – D shows the data for mean % correct responding for the 3 treatment groups divided into delay times (0 – 10 seconds). A repeated measures ANOVA on delay time (0, 2, 5 and 10 seconds) on the first 3 sessions after final withdrawal in the 2-CSRTT found a main effect of delay time ($F_{(3, 63)} = 10.89$ $p < 0.0001$) however no significant group x delay time interaction was found ($F_{(6, 63)} = 1.53$, $p = 0.182$) indicating that chronic ethanol treatment and withdrawal did not lead to differential levels of correct responding at the various delay times. There was a significant group x day interaction ($F_{(4, 42)} = 4.12$, $p = 0.008$.) Further investigation revealed that there was a significant group effect ($F_{(2, 21)} = 4.29$, $p = 0.0027$) in which post hoc tests revealed that RWD rats showed significantly lower percentage of correct responding during the first three sessions after the final withdrawal compared with CON rats ($p = 0.0032$) but not compared to SWD rats ($p = 0.13$) at all delay times.

A repeated measures ANOVA on delay time for the 3 sessions prior to reaching criterion (days -2 to 0) found a main effect of delay time ($F_{(3, 63)} = 4.96$ $p = 0.01$) however no significant group x delay time interaction was found ($F_{(6, 63)} = 1.38$, $p = 0.26$) indicating there was no effect of ethanol withdrawal on levels of correct responding at the various delay times. There was a significant group x day interaction ($F_{(4, 42)} = 6.014$, $p = 0.001$.) Further investigation revealed that there was no significant group effect ($F_{(2, 21)} = 0.323$, $p = 0.728$) which indicated that ethanol withdrawal did not significantly affect correct responding after long term withdrawal.

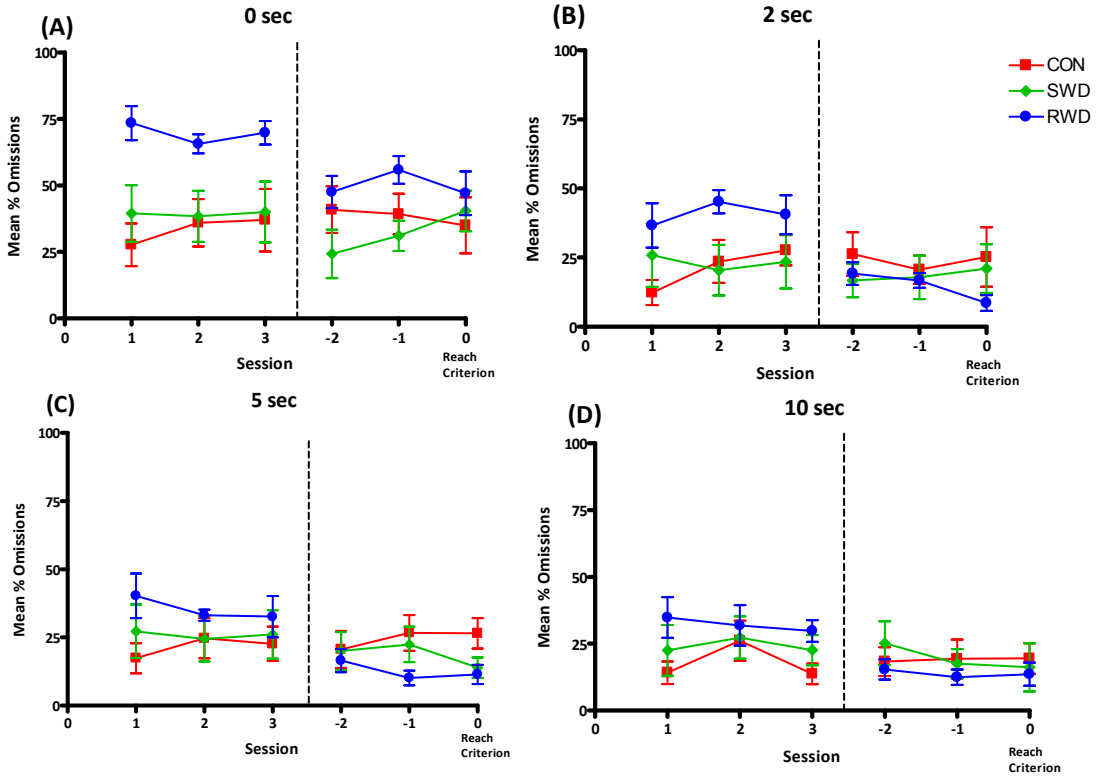


Fig 4.13: Mean % omissions in 2-CSRTT where training took place after chronic ethanol treatment was administered: Performance measured on the first 3 days of testing (Days 1 - 3) after the final withdrawal from ethanol and the last 3 days before reaching criteria (Days -2 - 0) post-withdrawal from ethanol. Graphs showed omissions at (A) 0 sec ITI, (B) 2 sec ITI, (C) 5 sec ITI and (D) 10 secs.

4.5.7. Omissions:

Figure 4.13 A-D shows mean omissions committed by the 3 treatment groups for the 4 delay times (0-10 seconds). Repeated measures ANOVA on delay time during the first 3 sessions after the final withdrawal found a main effect of delay time ($F_{(3, 63)} = 13.19$ $p < 0.0001$) however no significant group x delay time interaction was found ($F_{(6, 63)} = 1.55$, $p = 0.175$) indicating there was no effect of ethanol withdrawal on omissions at various delay times.

There was a main effect of day ($F_{(2, 42)} = 25.75$, $p < 0.0001$) and a significant group x day interaction ($F_{(4, 42)} = 3.98$, $p = 0.008$) was found. Further investigation revealed a significant group effect ($F_{(2, 21)} = 4.35$, $p = 0.026$) and post hoc tests revealed that RWD rats showed higher levels of omissions compared to CON rats ($p = 0.032$) but not compared to SWD rats ($p = 1.0$). This finding indicated that repeated ethanol

withdrawal led to attentional deficits during the early stages after the final ethanol withdrawal.

Analysis of the last 3 sessions prior to reaching criterion (days -2 to 0) found a main effect of delay time ($F_{(3, 63)} = 6.21$, $p = 0.004$) however no significant group x delay time interaction was found ($F_{(6, 63)} = 1.99$, $p = 0.11$) indicating there was no effect of ethanol withdrawal on omissions at various delay times.

There was a main effect of day ($F_{(2, 42)} = 41.96$, $p < 0.0001$) and a significant group x day interaction ($F_{(4, 42)} = 5.57$, $p = 0.001$) was found. However, further investigation found no significant group effect ($F_{(2, 21)} = 0.318$, $p = 0.731$) indicating that the attentional deficits observed during the early stages after the final ethanol withdrawal did not persist with extended 2-CSRTT training.

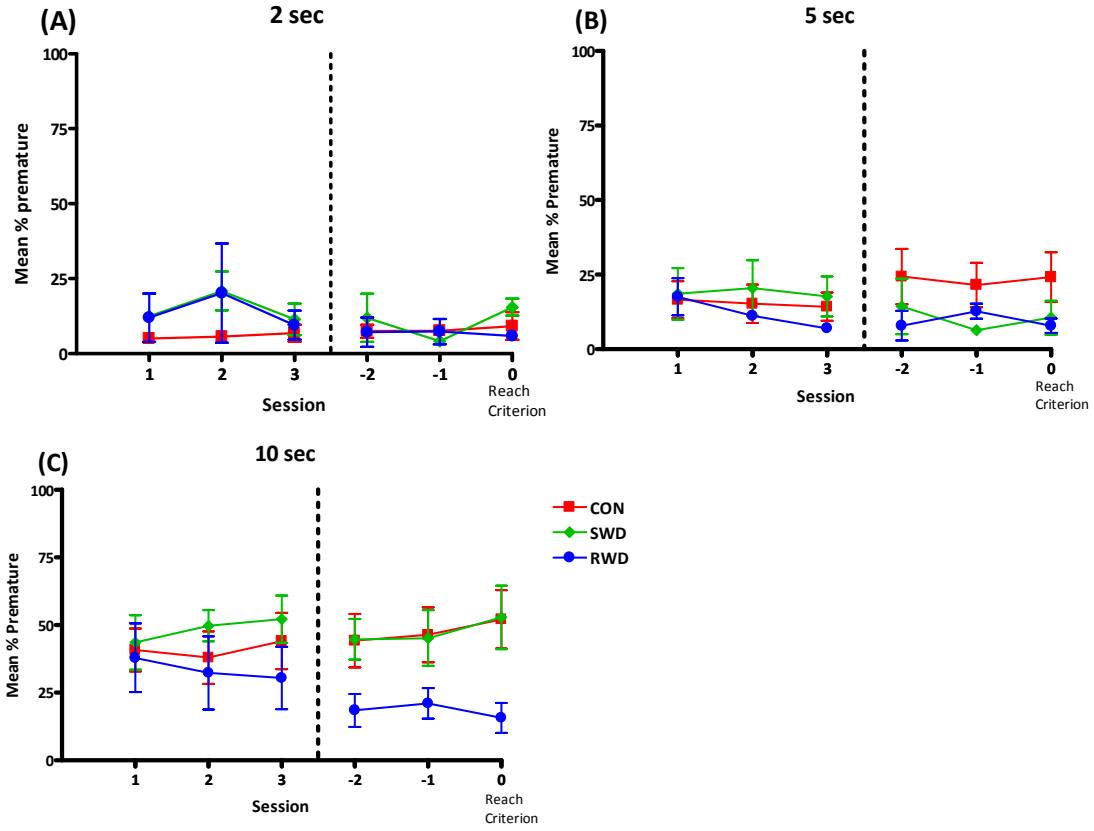


Fig 4.14: Mean % premature responses in 2-CSRTT where training on 2-CSRTT took place after chronic ethanol treatment was administered: Performance measured on Days 1 - 3 and Days -2 - 0 post-withdrawal from ethanol. Mean premature responses at (A) 2 sec ITI, (B) 5 sec ITI, (C) 10 sec ITI.

4.5.8 Premature responding:

Figure 4.14 A-C depicts the mean percentage premature responses committed by the 3 treatment groups, separated by delay times (0-10 seconds). A repeated measures ANOVA conducted on the first 3 sessions after protracted withdrawal on premature responding found a significant main effect of delay time ($F_{(2, 42)} = 27.6$, $p < 0.001$), which demonstrated that premature responding increased with increasing delay times, but no significant group x delay time x session interactions were observed ($F_{(4, 84)} = 0.441$, $p = 0.691$). No significant group effects were found ($F_{(2, 21)} = 1.36$, $p = 0.28$). Hence, repeated withdrawal from ethanol had no significant effects on premature responding on the 2-CSRTT during the early stages of protracted withdrawal.

Analysis of the last 3 days before reaching criterion on premature responding revealed a main effect of group ($F_{(2, 21)} = 6.46$, $p = 0.007$) with repeatedly withdrawn rats making significantly fewer premature responses than CON rats ($p = 0.036$) and SWD ($p = 0.017$) indicating repeated ethanol withdrawal resulted in less premature responding, particularly at the longer delay times. Single withdrawal from ethanol does not differ significantly from control group in premature responding ($p = 0.76$). These data suggest that single withdrawal from ethanol does not result in higher impulsivity levels whereas repeated ethanol withdrawal, contrary to the original hypothesis of these sets of experiments, may lead to lower levels of impulsive responding compared to baseline (as demonstrated by premature responding of control animals).

4.5.9. Conclusions:

The present study set out to examine the effects of repeated ethanol withdrawal on 2-CSRTT performance whereby ethanol withdrawal occurred prior to 2-CSRTT training and testing. This method was conducted in order to exclude the time period of ethanol administration from interrupting 2-CSRTT training and testing.

Using the identical 2-CSRTT experimental procedures of the previous experiments 1 and 2, with variable delay times, repeated ethanol withdrawal resulted in significant deficits correct responding during the initial stages of protracted withdrawal (days 1-3). However, with extended training sessions (days -2 to day 0), these attentional deficits were surmounted and at longer imposed delay times, repeatedly withdrawn rats showed superior correct responding (with the exception of performance at 0 second delay time). Singly withdrawn rats did not significantly differ from controls in their attentional performance nor impulsivity levels.

The poor initial attentional performance (as measured by correct responding) exhibited by RWD rats at 0 second delay time could be accounted for a high percentage of omissions as the percentage of premature responses was low (below 25%) at both 2 and 5 second delay times. This finding suggests that repeated ethanol withdrawal disrupted attentional performance when attentional load was most demanding (i.e. at 0 second delay time). Premature responses were attenuated with extended training.

The previous experiment in this current investigation (experiments 2) found that repeated ethanol withdrawal did not significantly impair 2-CSRTT performance.

This present finding suggests that repeated ethanol withdrawal results in attentional deficits if repeated withdrawal occurred prior to learning the task. This finding indicated that ethanol withdrawal disrupted learning but given there was no impairment of the 2-CSRTT performance in experiment 2, ethanol withdrawal does not impair previously acquired knowledge.

4.6. Experiment 4: Effects of RWD on 2-CSRTT performance using a fixed inter trial interval and variable inter trial intervals.

In the previous experiment (experiment 3), repeated ethanol withdrawal prior to 2-CSRTT training was found to result in attentional deficits, not observed when ethanol withdrawal occurred after 2-CSRTT training. A possible explanation for the lack of withdrawal effects on impulsive behaviour may be that exposure to variable delay times in previous experiments may have trained rats to be behaviourally flexible, masking any differences in performance that may have arisen.

This current experiment was conducted to investigate whether 2-CSRTT performance altered as a consequence of ethanol withdrawal a fixed 2 sec delay time during acquisition of the 2-CSRTT, the effects of ethanol treatment on task performance as well as post withdrawal effects on performance.

In order to test behavioural flexibility, variable delay probe trials were introduced on post withdrawal days 7, 14 and 21 to assess 2-CSRTT performance. An in-depth training procedure was described previously in section 4.2.5.

Experiment 4 investigated the effects of repeated ethanol withdrawal on 2-CSRTT performance, identical to experiment 1 and 2 with the exception of imposed delay times. In experiments 1, 2 and 3, the delay times varied between 0,2,5 and 10 seconds and the presentation of each delay time was selected in a pseudo-random order therefore rats were not able to predict the next delay, hence it was possible that rats were trained to be more behaviourally flexible as well as increasing their sustained attention. Experiment 4 involved training rats to criterion (3 consecutive training days

using 1 sec duration cue light stimulus, discrimination accuracy ≥ 70%) with the presentation of a fixed delay time of 2 seconds. Ethanol liquid diet was administered and rats continued 2-CSRTT daily sessions with a fixed delay of 2 seconds throughout chronic ethanol administration. Rats experienced a final withdrawal and 2-CSRTT performance was assessed throughout the withdrawal period using the same experimental parameters with the exception of days 7, 14 and 21 when the rats were subjected to a series of probe trials. During the probe trials, rats were presented with variable delay times, identical to the discrete trials in experiments 1,2 and 3 in which rats were required to wait 2,5, 10 or 15 seconds prior to the presentation of the cue light stimulus in order to respond for a food reinforcement. This current experiment tested the effects of repeated ethanol withdrawal on behavioural flexibility.

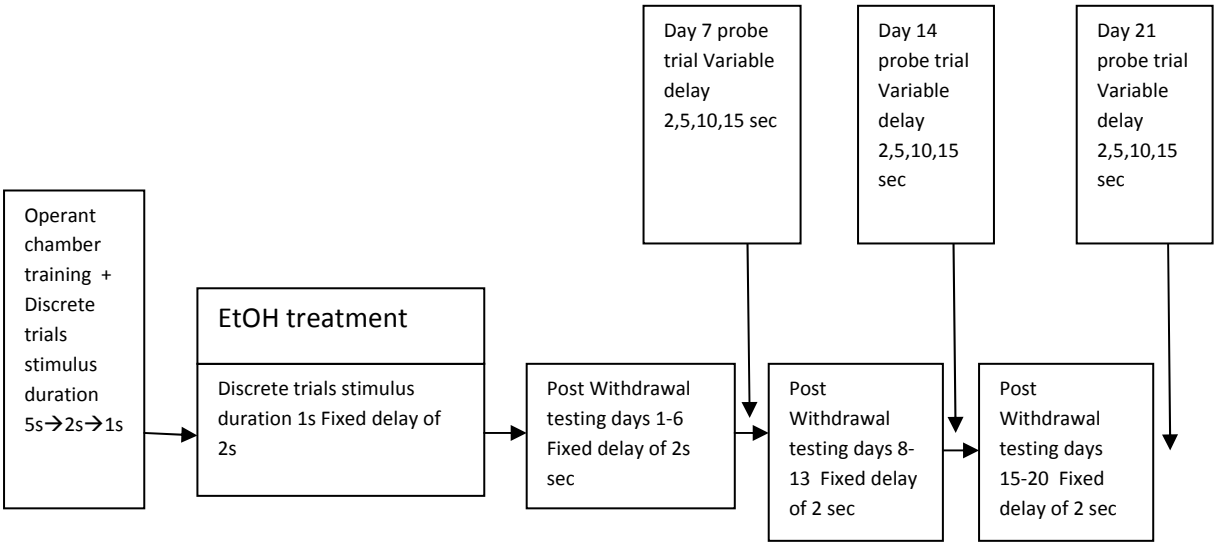


Figure 4.15: Experimental progression of Experiment 4.

4.6.1. Subjects:

Twenty-four male Lister hooded rats (Harlan, UK) weighed 135 – 150g at the start of the experiment. Acclimatisation and housing conditions were identical to those detailed in chapter 2, section 2.2.2.

4.6.2. Chronic ethanol treatment:

Chronic ethanol liquid diet administration was conducted in a manner identical to that described in chapter 2, section 2.2.1

4.6.3 Behavioural Procedure:

Behavioural procedure was identical to that of experiment 1 with the exception of using a fixed 2-second delay time. 2-CSRTT performance was assessed throughout chronic ethanol treatment to observe the effects of ongoing chronic ethanol intake on 2-CSRTT performance and continued for 3 weeks after the final withdrawal. Probe trials used variable delay times of 2, 5, 10 and 15 seconds and were performed on post withdrawal days 7, 14 and 21.

4.6.4. Results:

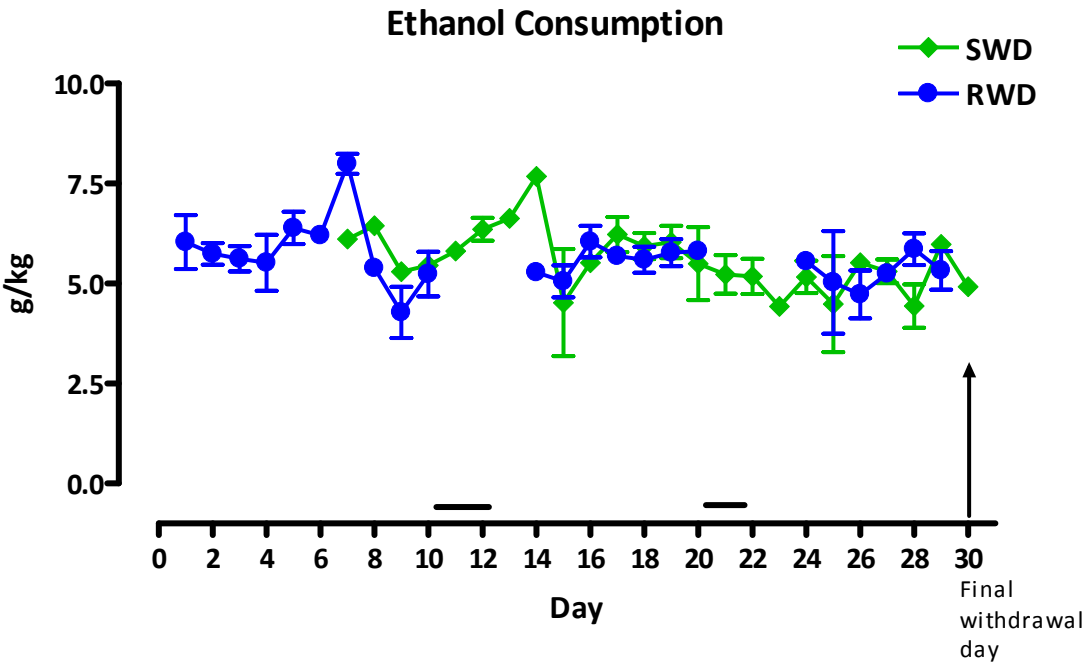


Fig 4.16: Mean ethanol consumption: Ethanol consumption is expressed as g per kg of body weight per day of treatment. Data represent the mean ± SEM. Arrow indicated the final withdrawal. During the last week of treatment, SWD and RWD rats consumed an average of 5 g/kg/day of ethanol.

4.6.5 Ethanol Consumption:

As can be seen in **Figure 4.16**, there were no group differences in ethanol consumption ($F_{(1, 6)} = 0.004$, $p = 0.95$). However, these rats did show very low levels of alcohol consumption in comparison to previous studies conducted in this laboratory.

4.6.6. 2-CSRTT performance during chronic ethanol treatment:

2-CSRTT performance assessed for the duration of chronic ethanol treatment did not reveal any significant differences between the treatment groups for correct responding (stimulus accuracy) ($F_{(2, 18)} = 0.55$, $p = 0.59$), discrimination accuracy ($F_{(2, 20)} = 1.74$, $p = 0.2$), omissions ($F_{(2, 18)} = 1.28$, $p = 0.31$), or premature responding ($F_{(2, 18)} = 0.295$, $p = 0.75$). Hence, ethanol consumption did not disrupt 2-CSRTT performance. Chronic ethanol consumption and intermediate episodes of withdrawal did not disrupt the latencies for correct ($F_{(2, 18)} = 1.48$, $p = 0.25$) and premature responses ($F_{(2, 14)} = 2.09$, $p = 0.16$).

4.6.7. Conclusions: 2-CSRTT performance using a fixed delay time was not affected by chronic ethanol treatment. Ethanol consumption in this current experiment was lower than in previous experiments. The low ethanol levels could have accounted for the lack of effects of chronic ethanol consumption on 2-CSRTT performance.

4.6.8. Post withdrawal effects on 2-CSRTT performance.

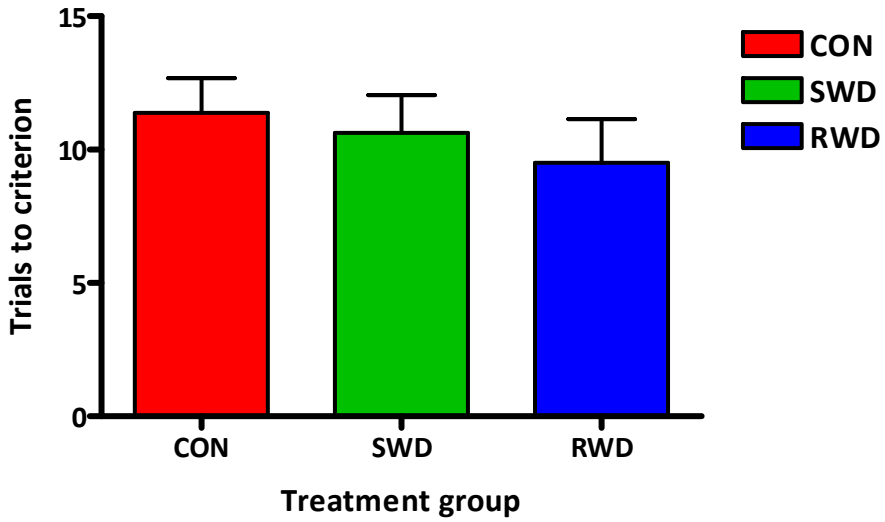


Fig 4.17: Mean number of sessions to criterion after final withdrawal from ethanol.

Statistical analysis of the number of sessions to criterion using a one-way ANOVA found no significant main effect of group ($F_{(2, 21)} = 0.47$. $p = 0.63$) indicating that repeated ethanol withdrawal did not significantly impair rats' learning of the 2-CSRTT.

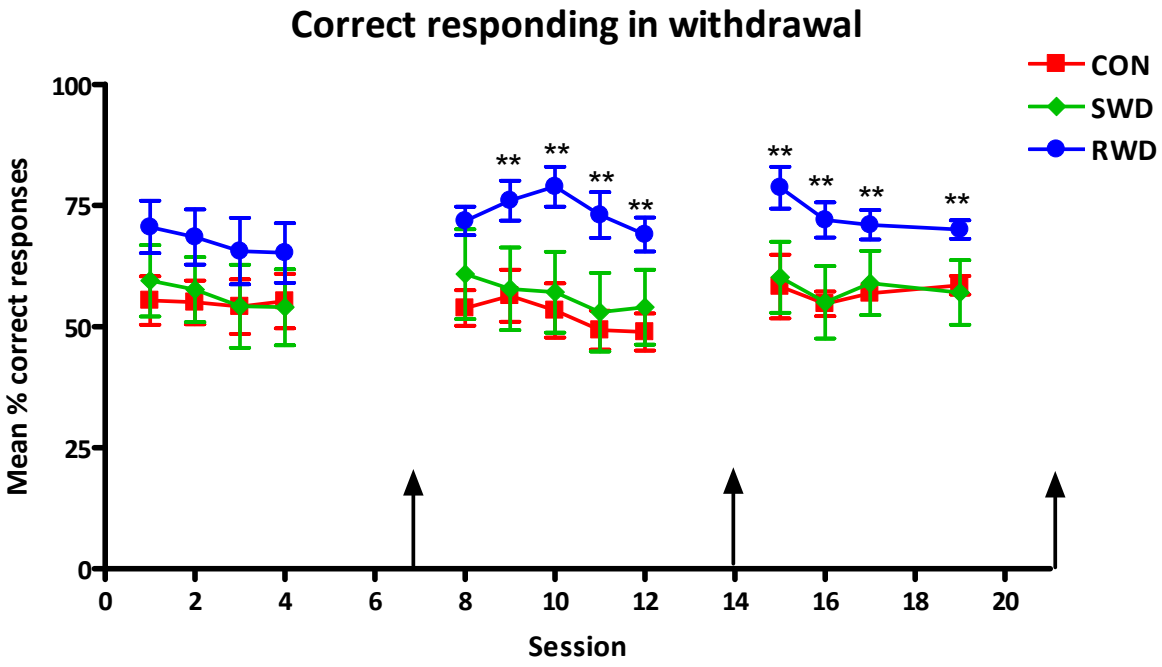


Fig 4.18: Mean % correct responding in 2-CSRTT with 2 sec fixed delay measured in withdrawal: Performance measured over 20 days after withdrawal from ethanol. 2-CSRTT used a 2 sec fixed delay time. Black arrows represent the time lines of the probe trials.

4.6.9. Correct responding:

Figure 4.18 shows the mean and SEM of correct responding in 2-CSRTT using a fixed 2-second delay time, assessed after the final withdrawal. A single factor repeated measures ANOVA found no main effect of group ($F_{(2, 21)} = 1.47$, $p = 0.25$) or session ($F_{(3, 63)} = 1.24$, $p = 0.30$) indicating that 2-CSRTT performance assessed prior to probe trial exposure was not affected by repeated ethanol withdrawal, nor did performance improve with session number during this period.

Analysis of correct responding after the 1st probe trial (sessions 8-12) found no main effect of session ($F_{(4, 84)} = 1.92$, $p = 0.16$) but a significant group difference was observed ($F_{(2, 21)} = 4.52$, $p = 0.023$). Further investigation showed that RWD rats exhibited higher levels of correct responding after the 1st probe trial than CON group ($p = 0.01$) and SWD group ($p = 0.034$).

Analysis of correct responding after the 2nd probe trial (sessions 15-19) found no significant main effect of session ($F_{(3, 63)} = 0.939$, $p = 0.38$). However, there was a

significant main effect of group ($F_{(2, 21)}$ = 4.86, p = 0.018), further analysis of which revealed RWD rats showed superior correct responding compared with both CON (p = 0.013) and SWD (p = 0.014) groups.

These results indicate that repeatedly withdrawn rats were superior in their 2-CSRTT performance than control animals and singly withdrawn rats, particularly after experiencing a probe trial session. Taken together, these findings indicate that RWD rats demonstrated significantly superior performance than control and SWD rats after exposure to the first and second probe trial sessions.

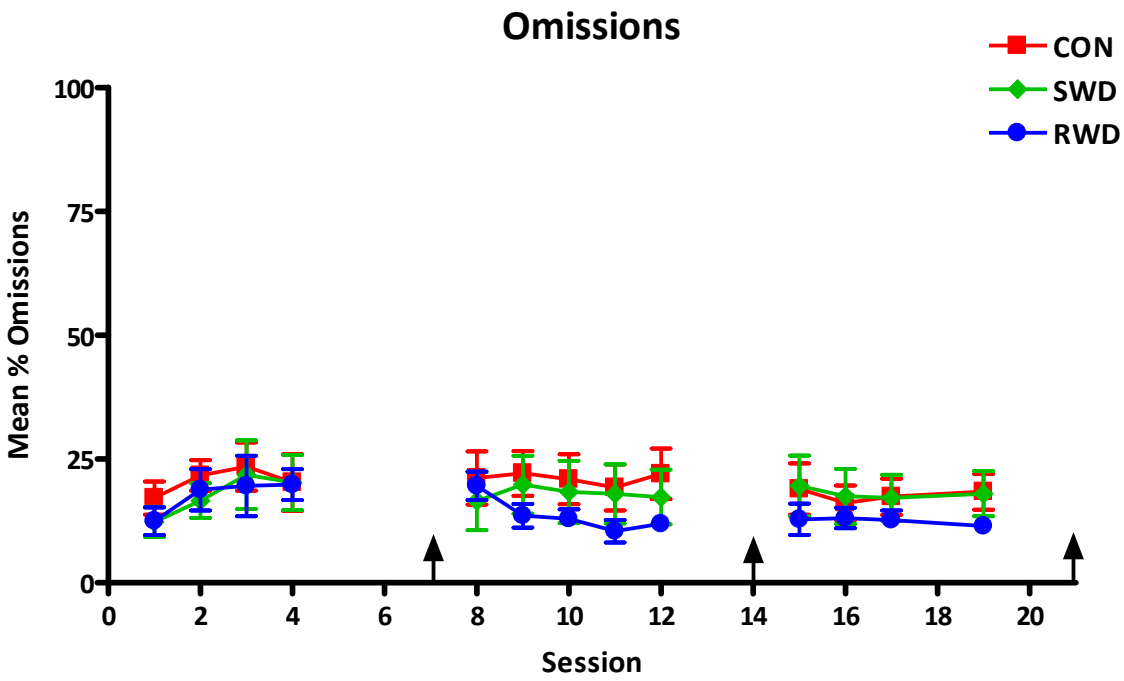


Fig 4.19: Mean % omissions in 2-CSRTT with 2 sec fixed delay measured in withdrawal: Performance measured over 20 days after withdrawal from ethanol. Black arrows represent exposure to probe trial sessions.

4.6.10. Omissions:

Figure 4.19 shows the mean and SEM of omissions in 2-CSRTT using a fixed delay time. A repeated measures ANOVA conducted on the sessions preceding exposure to probe trials found a significant main effect of session number ($F_{(3, 60)}$ = 4.62, p = 0.013). Further analysis found omissions increased with session number. There were no significant group differences ($F_{(2, 20)}$ = 0.23, p = 0.8)**.** Analysis of omissions after the first probe trial session found no significant main effects of session ($F_{(4, 80)}$ = 1.301, p = 0.28)

or of group ($F_{(2, 21)} = 3.96$, $p = 0.035$). Similarly analysis after 2nd probe trial sessions found no main effect of session ($F_{(3, 63)} = 0.59$, $p = 0.54$) or of group ($F_{(2, 21)} = 0.784$, $p = 0.47$).

These findings indicate that there were no effects of repeated ethanol withdrawal on omissions in the 2-CSRTT prior to or after exposure to probe trials.

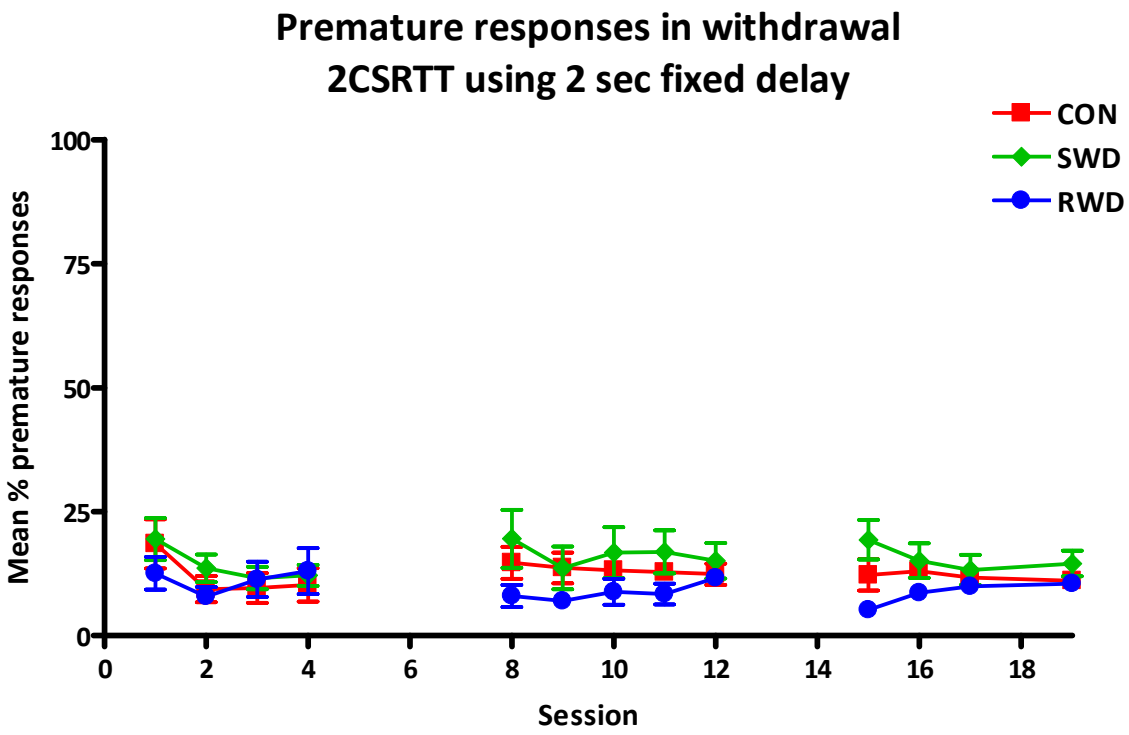


Fig 4.20: Mean % premature responses in 2-CSRTT with 2 sec fixed delay measured in withdrawal: Performance measured over 20 days after withdrawal from ethanol. 2-CSRTT used a 2 sec fixed delay time. Black arrows represent exposure to probe trial sessions.

4.6.11. Premature responding:

Figure 4.20 shows the mean and SEM of premature responses on the fixed delay 2-CSRTT. A repeated measures ANOVA conducted on the sessions prior to probe trial exposure found a main effect of session ($F_{(3, 51)} = 3.99$, $p = 0.013$) in which further analysis found premature responding decreased with session number. There were no significant group differences ($F_{(2, 17)} = 0.613$, $p = 0.55$) and no group x session interactions ($F_{(6, 51)} = 0.915$, $p = 0.49$).

No main effect of session was found after the first probe trial session ($F_{(4, 72)} = 0.613$, $p = 0.53$) or group ($F_{(2, 18)} = 1.46$, $p = 0.26$). After the 2nd probe trial session, there was no significant main effect of session ($F_{(3, 57)} = 0.17$, $p = 0.81$) and there was a tendency towards a group effect ($F_{(2, 19)} = 3.26$, $p = 0.06$) indicating RWD rats showed less premature responding compared to CON and SWD rats. However, this comparison did not reach significance.

These findings demonstrate premature responding decreased across session numbers. However, after the first and second probe trial sessions, this effect was not evident. A group effect, not previously observed began to emerge after the 2nd probe trial session, whereby RWD rats tended to show less premature responding than CON and SWD rats, an effect which did not reach significance.

4.6.12. Conclusions:

A caveat must be stated at the outset of concluding the results of the present experiment; ethanol consumption of alcohol-fed rats in this experiment was exceptionally low, therefore moderate to severe withdrawal symptoms after ethanol withdrawal, was not to be expected.

There were, nonetheless, some interesting findings from this current experiment. Although there were no significant difference between the treatment groups during the initial stages of withdrawal (prior to exposure to the probe trials), RWD rats showed superior 2-CSRTT performance (high correct responding) compared to CON and SWD rats. Repeated withdrawn rats also demonstrated lower premature responses but this measure did not reach statistical significance. This unexpected finding, contrary to the original hypothesis, suggested that repeated episodes of ethanol withdrawal did not disrupt attentional processes (correct responding) nor did it increase impulsive responding. However, it was possible that rats did not achieve blood ethanol concentrations during chronic ethanol treatment sufficient to generate withdrawal symptoms which may have impinged on the successful 2-CSRTT performance.

4.7. 2-CSRTT performance post-ethanol withdrawal using variable delay times in probe trials.

Rats exposed to variable delay times in the 2-CSRTT probe trials on post withdrawal days 7, 14 and 21 failed to learn the task. All rats responded to stimulus presentation at the 2-second delay time and performance was similar to the post withdrawal test sessions in between the probe sessions. However, as rats had not been exposed to variable delay times prior to the introduction of probe trials, rats did not learn to respond to 5, 10 or 15 second delay times. Probe trials did not affect 2-CSRTT performance on days during which fixed delay had been used. The introduction of probe trials in this current experiment, in which rats had been extensively trained using a fixed, 2-s delay, severely disrupted performance, and few rats were able in which rats had been extensively trained using a fixed 2-second delay, severely disrupted 2-CSRTT performance and few rats were able to acquire the rules of the task. For this reason, analyses of probe trial data were not found to be meaningful.

4.8. Discussion:

These current experiments examined the consequences of repeated ethanol withdrawal on motor impulsivity in rats. Previous experiments conducted in other laboratories investigating the link between impulsivity and alcohol consumption in rodent models primarily focused on tests of cognitive impulsivity. However, the 2-CSRTT assessed the effects of ethanol withdrawal on motor impulsivity which is thought to be mediated by a different brain pathway to cognitive impulsivity. Although rats with previous alcohol withdrawal experience demonstrated an unaltered ability to withhold a prepotent response, a deficit was observed in their attentional capacities which impaired their performance on the 2-CSRTT.

However, there is a limitation to the findings of this current study; impaired attentional capacity (using correct responses and omissions as indices) in RWD rats was seen in 2-CSRTT performance if ethanol withdrawal was imposed prior to training and testing on the 2-CSRTT. If ethanol withdrawal was imposed after the RWD rats had acquired the task, no difference in task performance was observed.

Furthermore chronic ethanol withdrawal did not significantly impair 2-CSRTT performance when tested using a fixed 2-second delay time. In withdrawal, prior to being subjected to probe trials, there were no significant differences in performance measures between the groups. However, after the exposure of the first and second probe trial sessions, the repeatedly withdrawn rats demonstrated superior attentional performance and there were no significant differences in impulsive responding compared with CON or SWD rats. Another constraint to the findings of experiment 4 was that the rats' ethanol consumption was exceptionally low, which resulted in mild rather than moderate or severe withdrawal symptoms. This constraint led to difficulty in the interpretation of the current results as there was little difference between the groups however, repeatedly withdrawn rats subsequently demonstrated superior performance after exposure to probe trial sessions compared with control and singly withdrawn rats. One interpretation of these results could be that repeatedly withdrawn rats were faster at acquiring the task with practice and this was reflective of the findings in experiment 3, in which RWD rats demonstrated superior attentional performance only upon reaching criterion. Nevertheless, there were no significant group differences in the number of trials to criterion when the rats were trained prior to receiving ethanol, yet RWD rats which received ethanol before training on the 2-CSRTT required significantly more trials to criterion, suggesting evidence of a learning deficit as a consequence of repeated ethanol withdrawal.

The current study found little evidence that repeated ethanol withdrawal in rats resulted in increased impulsivity which may be reflected in human studies by Townshend and Duka (Townshend and Duka 2005) who reported that female binge drinkers were impaired their ability to withhold a prepotent response but male bingers were not impaired in this task. This current study used male rats and this study's current findings correlate with Townshend and Duka's reported findings. It would have been interesting to investigate whether female rats subjected to repeated ethanol withdrawal demonstrated the behavioural deficits in impulsive responding as reported in humans.

Although this present study did not find that ethanol withdrawal increased motor impulsivity, it appears the correlation between alcohol and impulsivity has been

commonly reported in humans and animals. For example, chronic alcoholism is associated with impulse control deficits, attentional deficits and impaired judgement (Parsons 1987; Oscar-Bernman 1993). It has also been proposed that chronic alcohol consumption lead to changes in brain structure which may reduce behavioural control and this decreased control of behaviour may lead to further alcohol abuse and neurodegeneration (Crews, Collins et al. 2004). In a human questionnaire study by Nagoshi and colleagues (Nagoshi, Wilson et al. 1991) impulsivity was significantly correlated with higher levels of self-reported alcohol use. In a clinical study using the go / no go task by Noel et al (Noel, Van der Linden et al. 2007) alcoholics showed a deficit in their ability to withhold a prepotent response. These reported findings in humans are supported by studies in animal models of alcohol dependence, which have found that high-alcohol preferring mice are more impulsive than low-alcohol preferring mice on the delay discounting task (Wilhelm and Mitchell 2008; Oberlin and Grahame 2009). However, as mentioned previously, there are many varieties of impulsivity (Evenden 1999) and although studies have correlated cognitive impulsivity with chronic alcohol use, there is evidence that motor impulsivity may be mediated by different brain pathways (Dalley, Mar et al. 2008). There is evidence of cognitive impulsivity being mediated by the nucleus accumbens (Cardinal, Pennicott et al. 2001; Cardinal, Winstanley et al. 2004) whereas motor impulsivity may be associated with prefrontal cortical areas (Jentsch and Taylor 1999; Christakou, Robbins et al. 2001; Chudasama, Passetti et al. 2003).

In line with previous studies, the current results indicate that repeated ethanol withdrawal led to learning deficits compared with singly withdrawn and control animals. Previous studies conducted in this laboratory have found repeated ethanol withdrawal spares spatial learning in the Barnes maze (Borlikova, Elbers et al. 2006). Repeated ethanol withdrawal rats also showed a delayed learning to lever press for a food reward, although this result did not reach statistical significance (Stephens, Brown et al. 2001). Negative patterning was also impaired as a consequence of repeated ethanol withdrawal (Borlikova, Elbers et al. 2006). Negative patterning discrimination involves configural learning (Rudy and Sutherland 1989). In the negative patterning task, the subject is rewarded for responding to either stimulus A or stimulus

B when presented alone but not rewarded if A and B are presented in compound (A+, B+, AB-). Borlikova et al (Borlikova, Elbers et al. 2006) found no effect of repeated ethanol withdrawal on the acquisition of single element training, however during compound stimulus presentation, RWD rats were slower in reducing lever presses during the non-reinforced compound stimulus than CON and SWD groups. This finding indicates that repeated withdrawal from ethanol, in addition to delaying learning in 2-CSRTT, interferes with acquisition of lever pressing on a VI60s schedule, also delayed rats' learning to suppress responding to a compound stimulus presentation in negative patterning discrimination. Furthermore, repeated ethanol withdrawal resulted in deficits in learning associations between neutral stimuli and aversive events (Stephens, Brown et al. 2001). Although all the deficits which have arisen as a consequence of repeated experience of ethanol withdrawal concern different brain areas and involve different behaviours, taken together these findings indicate there is a general learning deficit produced by repeated ethanol withdrawal possibly as a consequence of neurobiological damage to the learning and memory brain circuits observed in animals and humans (Lukoyanov, Madeira et al. 1999; Moselhy, Georgiou et al. 2001; Obernier, White et al. 2002; Farr, Scherrer et al. 2005). Neurobiological studies by Nixon and Crews (Nixon and Crews 2002) have reported that ethanol inhibits brain neural stem cell neurogenesis which may contribute to learning deficits (Crews and Braun 2003).The neurodegeneration caused by chronic ethanol consumption may result in persistent learning deficits, for instance rats exposure to ethanol in a binge model demonstrate perseverative responses in reversal learning tasks compared with control rats (Obernier, White et al. 2002). These findings show that chronic ethanol is consistently associated with behavioural learning deficits which the results from the current study have also reported.

Conclusions:

In summary, the current study in 2-CSRTT found no effects of repeated ethanol withdrawal on motor impulsivity as measured by the novel 2 choice serial reaction time task. However, previous experience of ethanol withdrawal did give rise to attentional and learning deficits but only if learning occurred after chronic ethanol administration. Learning deficits were not observed if learning occurred prior to

chronic ethanol administration. Further studies might investigate whether repeated ethanol withdrawal leads to alterations in different types of impulsivity e.g. cognitive impulsivity in the delay-discounting task.

Chapter 5

The effects of repeated ethanol withdrawal on attentional set shifting

5.1. Introduction:

The prefrontal cortex has been implicated in various cognitive and executive functions including working memory (Brozoski, Brown et al. 1979; Granon, Vidal et al. 1994; Broersen, Heinsbroek et al. 1995; Delatour and Gisquet-Verrier 1999; Aultman and Moghaddam 2001; Floresco and Phillips 2001), attentional processes (e.g. attentional set shifting ability)(Olton, Wenk et al. 1988; Dias, Robbins et al. 1996; Bussey, Muir et al. 1997; Birrell and Brown 2000; Delatour and Gisquet-Verrier 2000; Granon, Passetti et al. 2000; Chudasama and Muir 2001; Chudasama and Robbins 2003), decision making (Ainslie 1975; Richards, Mitchell et al. 1997; Evenden 1999; Mobini, Chiang et al. 2000; Mobini, Body et al. 2002), inhibitory response control (Carli, Robbins et al. 1983; Granon, Passetti et al. 2000; Koskinen, Ruotsalainen et al. 2000; Dalley, McGaughy et al. 2001; Chudasama, Passetti et al. 2003) and the temporal integration of voluntary behaviour(Kolb, Buhrmann et al. 1994; Fuster 2000).

Heavy chronic alcohol intake has been found to result in physiological frontal lobe dysfunction such as decreased local cerebral metabolic rates for glucose in the medial-frontal area of the cortex in alcoholic patients (Gilman, Adams et al. 1990; Adams, Gilman et al. 1993). Studies also reported that chronic alcohol consumption led to impaired function of the medial frontal region of the brain, affecting tissue metabolic rates and neuropsychological correlates (using the Wisconsin Card Sorting test) (Adams, Gilman et al. 1993; Adams 1995). Other studies have suggested that severe alcoholism damages $GABA_A$ / benzodiazepine receptors in the frontal lobes, which may be a risk factor in the development of alcohol-related behaviours (Deckel, Bauer et al. 1995; Gilman, Koeppe et al. 1996). Furthermore, both electroencephalographic (EEG) and evoked potential studies support the presence of physiological brain changes in alcoholics, especially in the frontal lobe (Pribram 1973; Begleiter, Porjesz et al. 1980; Porjesz, Begleiter et al. 1980; Michael, Mirza et al. 1993; Bauer, O'Connor et al. 1994; O'Connor, Bauer et al. 1994; Cohen, Projesz et al. 1996). There is also evidence of structural abnormalities in the frontal lobe associated with alcoholism. For instance, in

a post mortem study by Harper and associates (Harper, Kril et al. 1987), chronic alcoholics showed a significant loss of brain tissue. Moreover, the number of cortical neurones in the superior frontal cortex in chronic alcoholic patients was significantly reduced in comparison to controls. Studies investigating the effects of alcohol on receptors in the frontal cortex have found that chronic alcoholism leads to increased density of NMDA receptors in the frontal cortex which may represent a stage of alcohol-induced chronic neurotoxicity (Volkow, Wang et al. 1993; Dodd, Kril et al. 1996; Freund and Anderson 1996; Gilman, Koeppe et al. 1996; Lewohl, Crane et al. 1997; Marchesi, Ampollini et al. 1997).

Although chronic alcoholics do not show deficits in general measures of intelligence (Tarter 1975b; Tarter 1980; Parsons 1987), detailed testing of alcoholic subjects do reveal deficits in cognitive flexibility, problem solving, visuo-motor coordination, learning, conditioning and memory (Jones and Parsons 1971; Jones and Parsons 1972; Parsons 1975; Tarter 1976; Butters, Cermak et al. 1977; Cala, Jones et al. 1978; Jenkins and Parsons 1979; Tarter 1980; Bergman 1985; Miller 1985; Acker 1986; Wilkinson 1987; Nicolas, Catafau et al. 1993; Beatty, Hames et al. 1996; Nixon and Bowlby 1996).

Chronic alcoholics showed deficits in tests designed to be sensitive to frontal lobe damage. These tests include the Porteus maze task, in which the subject must find the most direct route through a maze without entering blind alleys or crossing through lines; and the Wisconsin Card Sorting Test (WCST). The WCST (Milner 1963) is a neuropsychological assessment of attentional set shifting. Attentional set shifting refers to the switching between higher order modalities (e.g. from lines to shapes or from texture to odour) based on feedback in the form of reinforcement or the absence of reinforcement. The WCST requires subjects to learn the correct method of sorting a deck of cards, by means of trial and error based on feedback provided by the experimenter. Once the subject learns the rule that governs their responses, the rule is changed (but not explicitly stated by the experimenter to the subject), and the subject must sort the cards by another rule. Hence, the subject does not learn a specific response but the rule that governs their responses to a specific situation. The WCST involves a series of discriminations which test a subject's ability to acquire a rule, the ability to learn a new rule and disregard the old rule and the ability to demonstrate

reversal learning. In reversal learning, the subject must first learn to make a discrimination i.e. choosing a black object in a black-white discrimination problem, and then learn to reverse their choice – i.e. choose the white object.

Perseverative responding appears to be the most characteristic error in frontal patients (Barcelo, Sanz et al. 1997) in which subjects assign their attention to the incorrect classification criterion even when the subject has detected an error in their decision making, which appears to be related to the ability to shift cognitive set. Perseveration is considered to be an example of cognitive inflexibility.

Poor performance on the WCST has been reported to be due to frontal lobe damage (Drewe 1974). Several neuroimaging studies investigating the WCST performance have found activation of the dorsolateral PFC, using regional cerebral blood flow (rCBF) (Weinberger, Berman et al. 1986; Marenco, Coppola et al. 1993; Rezai, Andreasen et al. 1993; Catafau, Parellada et al. 1994). However, there is evidence of the involvement of other brain areas such as the parietal, medial temporal and hippocampal cortices in the WCST (Hermann, Wyler et al. 1988; Anderson, Damasio et al. 1991; Corcoran and Upton 1993; Mountain 1993; Upton and Corcoran 1995). In neuropsychological assessments, chronic alcoholic subjects performed worse than both the control group and brain damaged patients (Fitzhugh, Fitzhugh et al. 1960; Fitzhugh, Fitzhugh et al. 1965), a finding which has been supported by numerous studies using frontal lobe tests (Jones and Parsons 1971; Smith, Burt et al. 1973; Long and McLachlan 1974; Goldstein and Shelly 1980; Hill 1980; Parsons 1987; Sullivan 1993).

High alcohol consumption causes neurodegeneration which contributes to loss of executive functions mediated by the PFC (Crews and Boettiger 2009). Chronic alcohol consumption is associated with impaired judgement, poor insight, reduced motivation, attentional and impulse control deficits (Parsons 1987; Oscar-Bernman 1993). Crews and colleagues suggest that progressive increases in alcohol consumption lead to changes in brain structure that results in a loss of control over drinking behaviour, culminating in further alcohol abuse (Crews, Collins et al. 2004). There is evidence of general changes in brain function after repeated withdrawals from ethanol, for

example, rats show enhanced metabolic activity in the limbic and cortical brain areas after repeated withdrawal experience (Clemmesen, Ingvar et al. 1988).

Repeated episodes of ethanol withdrawal in rats have been reported to induce over-responding in a fixed interval paradigm in the period immediate before food reinforcement was available, suggesting that rats with previous experience of withdrawal may have displayed an inability to withhold inappropriate responding at the time of expected reinforcement (Borlikova, Elbers et al. 2006). This finding suggests a role for the prefrontal cortex in withdrawal, as this brain area in involved in response inhibition (Robbins 1996; Rubia, Smith et al. 2003; Aron, Robbins et al. 2004). Hence, it would be of great interest to investigate the effects of repeated ethanol withdrawal on attentional set shifting as a function of the PFC.

Attentional set shifting involves the ability to discriminate and response to a stimulus and is analogous to the cognitive flexibility required for successful performance on the WCST. It has been recognised that components of the WCST are related to the extradimensional shift test, derived from human and animal learning theory (Roberts 1988; Downes, Roberts et al. 1989). The subject forms an attentional set based on perceptual features of a stimulus in order to enhance the efficiency of processing relevant information, and to ignore irrelevant information. In animal studies, perceptual attentional set shifting is assessed using the intra-dimensional / extradimensional shift, originally devised by Birrell and Brown (Birrell and Brown 2000). Extradimensional shift required a subject to transfer attention in compound stimuli from one perceptual dimension to another based on changing reinforcement or feedback. Control tests include the ability to shift attention based on altered feedback within a dimension (i.e. reversal learning) and the ability to shift attention to novel exemplars of the same dimension (i.e. intradimensional shift) (Roberts 1988; Owen, Roberts et al. 1991).

Behavioural flexibility, an executive function which represents the ability to learn a new strategy while inhibiting the execution of a previous strategy (Ragozzino, Detrick et al. 1999), is necessary for successful adaptation to a changing environment (Kolb 1990). Behavioural flexibility is related to cognitive functions mediated by the

prefrontal cortex (PFC) in humans and primates (Milner 1982; Owen, Downes et al. 1990; Owen, Roberts et al. 1993; Dias, Robbins et al. 1996a; Dias, Robbins et al. 1997). Different regions of the PFC mediate different aspects of behavioural flexibility. For example, the dorsolateral PFC (DLPFC) is involved in inhibiting responses based on one dimension that was previously deemed correct, and learning to respond to a different dimension (Dias, Robbins et al. 1996a; Dias, Robbins et al. 1997). However, the DLPFC does not appear to be significantly involved in reversal learning (Dias, Robbins et al. 1996a), which may be defined as inhibiting a response. The orbitofrontal cortex (OFC) has been reported to mediate reversal learning in humans (Hampshire and Owen 2006) and rats, however lesions to this brain region were not found to impair attentional set shifting in the rat (McAlonan and Brown 2003) indicating that reversal discrimination and attentional set shifting are dissociable by brain area.

Despite anatomical differences between the human / primate brain and the rodent brain, functional homology can still be determined in the rodent brain as Kolb suggested that the medial wall cortex of the rat brain is “undifferentiated” prefrontal cortex and this area may mediate the same cognitive functions as in the dorsolateral PFC in primates (Kolb 1990). As the medial PFC of rodents and the dorsolateral PFC in primates share many functional similarities (Kesner 2000), it may be argued that if the rat demonstrates complex behaviour which, in primates, require the dorsolateral PFC, then the brain areas subserving these behaviours may be considered analogous to the dorsolateral PFC (Brown and Bowman 2002).

The intradimensional / extradimensional (IDED) task is a behavioural task originally devised by Birrell and Brown (Birrell and Brown 2000) to address the issue of rodent prefrontal cortical function, explicitly related to attentional set shifting and was devised to be similar in function to the WCST, used to detect frontal lobe damage in humans. In essence, the IDED task is a “two-choice discrimination attentional set shifting task where complex stimuli differ along several perceptual dimensions” (Birrell and Brown 2000) and was adapted from similar tasks used in primates (Iversen and Mishkin 1970; Roberts, De Salvia et al. 1994; Dias, Robbins et al. 1996a; Dias, Robbins et al. 1996b; Dias, Robbins et al. 1997) and humans (Nauta 1971; Downes, Roberts et al. 1989; Owen, Roberts et al. 1991; Gallagher, McMahan et al. 1999; Ferry, Lu et al.

2000). The IDED task consists of a series of compound perceptual discriminations which required animals to either maintain an attentional set and transfer behavioural responding from one pair of exemplars to another within the same perceptual dimension (intradimensional (ID) shift) or to shift an attentional set from one perceptual dimension (e.g. odour or medium) to another (otherwise known as extradimensional (ED) shift). This behavioural task also included several reversal discriminations, in which the animal was required to maintain an attentional set but learn a new stimulus-reward association.

The present study was designed to investigate the effects of repeated ethanol withdrawal and attentional set shifting ability in rats using the intradimensional / extradimensional set shifting task. Further investigation of the frontal areas involved in both intradimensional and extradimensional shifts were also conducted using the expression of the immediate early gene c-fos as a measure of activation. Based on experiments investigating the effects of repeated ethanol withdrawal on c-fos expression (see chapter 3) which found a general activation of the prefrontal cortical areas in rats with previous experience of ethanol withdrawal, we would predict deficits in attentional set shifting in repeatedly withdrawn rats as a consequence of the withdrawal-related activation of the prefrontal areas. In a recent study, Burnham and associates found increased Fos-like immunoreactivity in the medial and orbital frontal cortex of rats performing attentional shifts compared to rats performing control discriminations which suggests the differential recruitment of cortical brain areas associated with attentional set shifting (Burnham, Bannerman et al. 2010). Additionally, control rats which had been divided into either ID shift or ED shift would be predicted to demonstrate increased c-fos expression in the medial prefrontal cortex which would be associated with the ED shift as distinct from the ID shift.

Table 5.1: Definitions of the discriminations involved in IDED task

Discrimination	Definition	Primary brain areas involved
Simple discrimination	Digging bowls differ from each other in 1 dimension only (i.e. 2 bowls of sawdust, one is scented with lemon which is the positive food predictor, the other is scented with lavender which does not predict food reward.)	
Compound discrimination	Same correct and incorrect exemplars used in simple discrimination, but with the addition of an irrelevant 2nd dimension i.e. lemon in straws (+) and lavender in pipe cleaners (-)	
Reversal	Same exemplars as in compound discrimination but the exemplars previously incorrect are now correct, i.e. lavender (+) and lemon (-) in irrelevant digging material.	Frontostriatal circuits, particularly orbitofrontal cortex.
Intradimensional shift (IDS)	New set of exemplars but the new correct exemplar is in the same dimension as in previous discriminations (i.e. odour). Coriander (+) and cumin (-). New digging materials are still irrelevant.	
Extradimensional shift (EDS)	New set of exemplars but the previously irrelevant dimension (digging material) is now relevant and associated with food reward. So rats have to learn to ignore the previously relevant stimulus dimension odour), in favour of the newly relevant stimulus dimension (texture).	Medial prefrontal cortex, namely the infralimbic and prelimbic cortices.

5.2. Materials and Methods:

Experiment 1: Investigating the effects of repeated ethanol withdrawal on IDED task performance.

5.2.1. Subjects:

Thirty-six male Lister Hooded rats weighed approximately 150 g at the beginning of the experiment. Acclimatisation and housing conditions were identical to those detailed in chapter 2, section 2.2.2.

5.2.2. Chronic ethanol treatment:

Chronic ethanol treatment was administered as previously described in chapter 2, Section 2.2.1. All animals experienced a final withdrawal from liquid diet at 08:00 h and remained in their home cages with *ad libitum* access to food and water for 2 weeks after the experience of final withdrawal. Rats were then food restricted for one week (20 g / day / rat standard rat chow). During this time, rat chow was placed into the ceramic bowls that were subsequently used in the IDED task and placed into the home cages in order to reduce the confounding factor of neophobia of the digging bowls.

5.2.3. Task Design:

The intradimensional / extradimensional (IDED) task was designed so that both ID and ED acquisition stages were conducted using novel stimuli which were useful in reducing the possible confounding effects of stimulus novelty and furthermore, the only difference between the ID and ED acquisition is the previous relevant dimension. Rats were trained to dig for a food reward (half a honey nut Cheerio; Nestle, UK) and to make discriminations based on the odour or the texture of the digging material in which the bait was buried.

5.2.4. Behavioural Apparatus:

The test apparatus was a Perspex box (40 x 70 x 18 cm) with Perspex panels used to divide one third of the length of the box into 2 separate sections. The larger section represented the “waiting” compartment, the smaller section the test compartment. Sliding Perspex barriers prevented access to the experimental area into which two digging bowls were placed. Furthermore, access within the 2 smaller compartments in the test area could be blocked, thus allowing access to only one digging bowl. The function of the compartments prevented the rat being able to dig in both bowls before the bowls were removed from the experimental area. Without the dividers, a rat could move between the two digging bowls and quickly acquire the food from the 2nd bowl before being stopped. This difficulty was avoided by sliding the divider down as soon as the rat began to dig in one bowl, preventing access to the other bowl, following the procedure of Birrell and Brown (Birrell and Brown 2000).

5.2.5. Behavioural Training:

1. **Procedure:** Initially, the subject was allowed to explore the bowls by touch and smell. A dig was defined as such when the digging medium was significantly displaced. For the first four trials, the rat was permitted to recover the food reward from the bowl, even if it had initially dug in the incorrect bowl. After the first four trials, if the rat made an incorrect bowl choice, it was not permitted to recover the food reward from the baited bowl; hence, once the rat digs in either bowl, access to the other bowl was immediately denied.

The dimensions of odour and digging material were counterbalanced across rats, so an equal number of rats from each treatment group were exposed to each shift (i.e. odour to digging material, or digging material to odour).

2. **Habituation:** In order to acclimatise the subjects to the test equipment, rats were initially placed into the test box for 30 minutes and permitted to roam freely through the two compartments (waiting compartment and the testing compartment). After 30 minutes, the subject was confined to the waiting compartment (access to the test compartment was denied using dividers) whilst 2 ceramic bowls, both baited with a food reward and filled with unscented sawdust were placed in the test compartment. The dividers were raised and the subject was permitted access to both bowls and learned to dig in the bowls to retrieve food reward. Both bowls were re-baited every 10 minutes for a total of 1 hour.

Once rats were digging reliably, they were subsequently trained on two simple discriminations, presented to the subjects in the same order. Rats were tested on an odour discrimination and a digging material discrimination. Criterion performance in all discriminations was six consecutive correct trials.

3. **Testing:** Testing on the IDED task was conducted 1-2 days after habituation. The discriminations were presented in a fixed order for all subjects: namely a simple discrimination (**SD**) in which the presented digging bowls varied in only one characteristic, a compound discrimination (**CD**) in which a second, but irrelevant characteristic was introduced; a reversal of the compound

discrimination (**R1**); an intradimensional shift (**ID**) in which a novel set of exemplars were presented; a reversal of the ID discrimination (**R2**); an extradimensional acquisition (**ED**) with another set of novel exemplars and a reversal of the ED discrimination (**R3**).

Table 5.2: Exemplars used

Dimension	*Training set*	*Set 1*	*Set 2*	*Set 3*
Odour	Mint	Cinnamon	Thyme	Nutmeg
	Oregano	Cumin	Paprika	Cloves
Medium	Rubber bungs	Eppendorf lids	Sponges	Latex gloves
	Polystyrene packaging	Eppendorf tubes	Cotton swabs	Rubber bands

5.3. Experiment 2: Differential C-fos expression as a result of Intradimensional or Extradimensional stages of set shifting.

In order to investigate the differential induction of c-*fos* expression by the intradimensional shift and the extradimensional shift, rats were run through a modified sequence of discriminations on either IDS completion or EDS completion, with no reversal discriminations included. This stage was carried out approximately 7 days after completion of testing on the original IDED task. All presentations of dimensions were fully counterbalanced. Rats were presented with the same exemplars at each stage. All animals were tested on both simple and compound discrimination. A rat tested up to IDS was tested in the following order: Simple discrimination > Compound discrimination > IDS.

A rat tested up to EDS was tested in the following order: Simple discrimination > compound discrimination > EDS. In the final discrimination, all rats were presented with the same exemplars.

Rats were habituated to the test apparatus for 30 mins prior to exposure to any exemplars, after which, each exemplar was presented to the rat in pairs. Odours were

presented in sawdust, digging medium was on top of unscented sawdust. This was done to prevent rats developing neophobia to the exemplars.

Dimension	*Simple discrimination*	*Compound discrimination*	*IDS*	*EDS*
Odour	Tarragon	Tarragon	Coriander	Coriander
	Ginger	Ginger	Sage	Sage
Medium	Sawdust	Beads	Shredded paper	Shredded paper
	Sawdust	Cloth	Thin cardboard	Thin cardboard

Table 5.3: IDS vs. EDS. Exemplars used.

As with previous behavioural testing, rats reached criterion of 6 consecutive correct digs before advancing to the next discrimination. Once the subject completed the series of discriminations, it was returned to its home cage prior to transcardial perfusion 2 hours later.

5.3.1. Histology:

Two hours after the conclusion of behavioural testing, rats were deeply anaesthetised using an overdose of sodium pentobarbital and transcardially perfused. Histological methods used in this current experiment were identical to those previous detailed in chapter 3, section 3.2.3.

5.4. Results:

5.4.1. Chronic Ethanol consumption:

As depicted in **figure 5.4,** the mean ethanol consumption over the final 7 days of chronic ethanol treatment (following the last intermediate withdrawal in the RWD group) was 16.9-± 0.4 g ethanol per kg body weight per day in the SWD group and 18.9 ± 0.5 ethanol per kg body weight per day in the RWD group. A one-way ANOVA over this period revealed that there were no significant differences in ethanol intake across the 2 groups ($F_{1,10}$ = 3.795, p = 0.08).

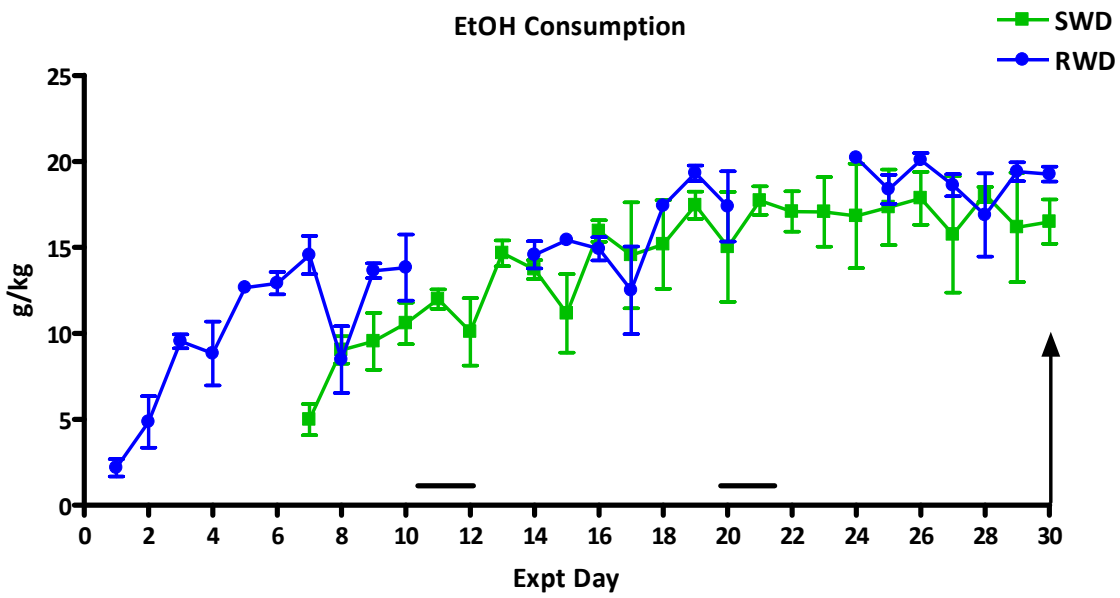


Fig 5.4: Mean± *SEM* ethanol consumption: Ethanol consumption expressed as g per kg of body weight per day of treatment. Arrow indicates the final withdrawal; intermediate withdrawal episodes in the RWD group took place on treatment days 11-13 and on treatment days 21 – 23. There was no overall difference between the groups in consumption ($p > 0.05$).

5.4.2. Intradimensional / extradimensional Task

Trials to criterion:

Figure 5.5 shows the number of trials to criterion at each stage of the task, in the order in which the discrimination stages were presented. From the graph, no differences were observed between the 3 treatment groups. A repeated measures ANOVA revealed a main effect of discrimination ($F_{6,198} = 35.5$, $p < 0.001$) further analysis revealed that rats required significantly more trials to criterion in the extradimensional shift than any other discrimination ($p < 0.001$). However, there were no significant group x discrimination interactions ($F_{2,33} = 1.4$, $p = 0.27$) and no significant group differences ($F_{2,33} = 0.382$, $p = 0.69$). The findings from the present study found that chronic ethanol consumption and withdrawal did not significantly affect successful performance on the IDED task, indicating that attentional processes involved in attentional set shifting and reversal learning were not hindered by repeated ethanol withdrawal.

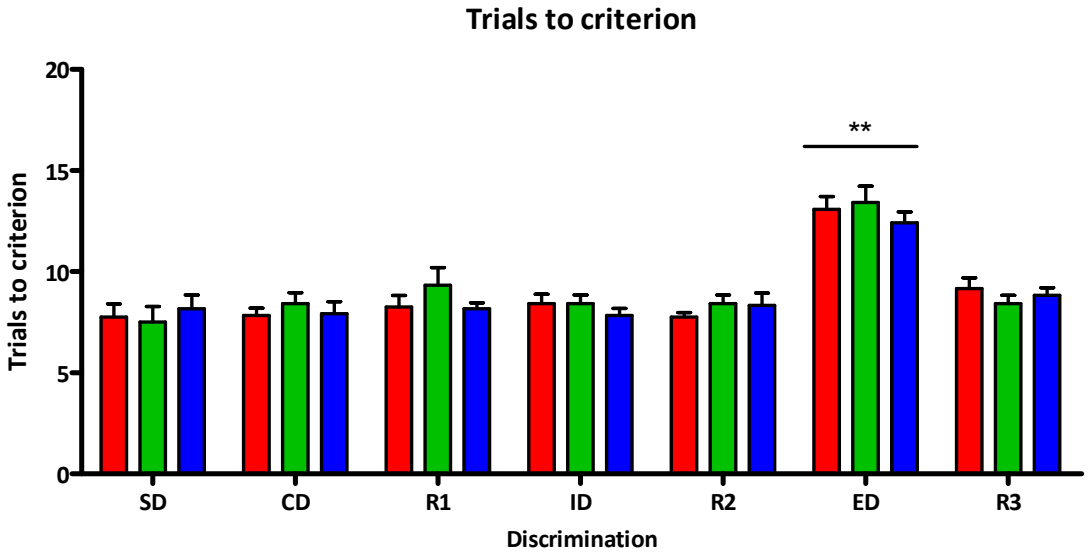


Fig 5.5: Trials to criterion in IDED task for the 3 treatment groups. Bar graph depicts mean number of trials to criterion on the IDED task. Red = control group, green = SWD group and blue = RWD group. Analysis revealed all rats required a significantly greater number of trials to criterion on the extradimensional shift ($p < 0.001$) compared to all the other discriminations; however, there were no significant group differences.

5.4.3. C-Fos Differentiation of the brain areas involved in Intradimensional / Extradimensional Task:

As no group differences were observed in the previous IDED task of this present study, the next aim was to determine the prefrontal areas activated by intradimensional and extradimensional shift. These current experiments were conducted only in control animals. Due to the lack of behavioural differences in SWD and RWD groups, immunohistochemistry was not assessed in these rats.

Figure 5.6 shows the number of trials to criterion for ID discrimination and ED discrimination. A one-way ANOVA the trials to criterion for ID and ED shifts found that rats required significantly more trials to criterion in the extradimensional shift that in the intradimensional shift ($F_{1,10} = 12.5$, $p = 0.005$).

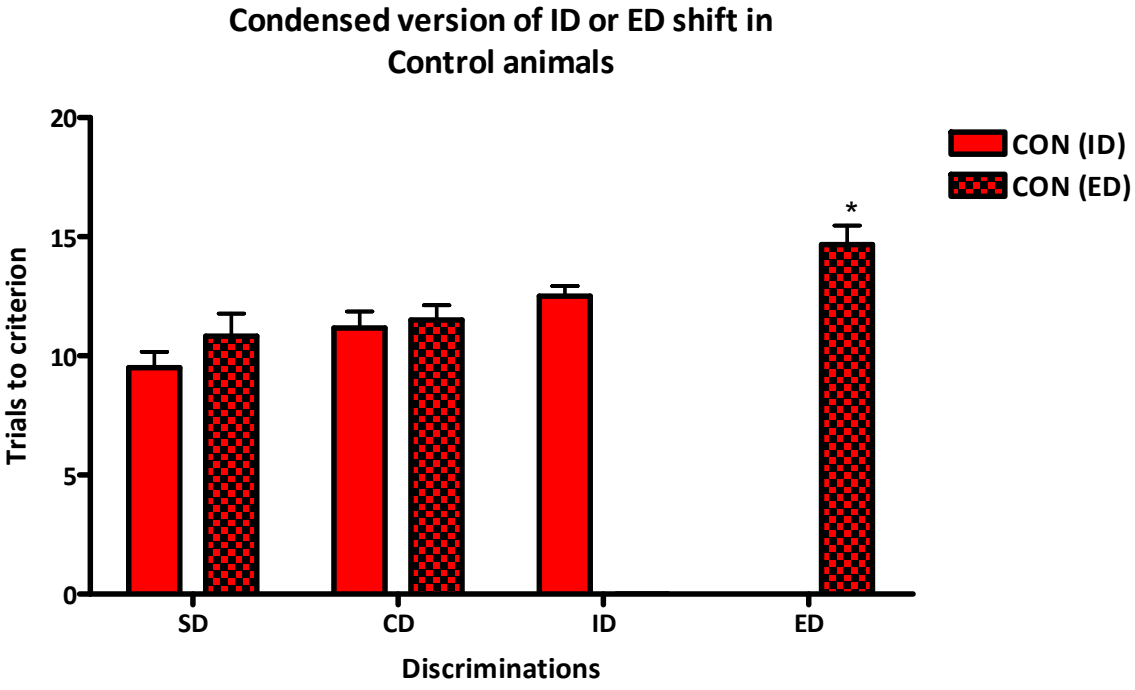


Fig 5.6: Trials to criterion in a condensed version of IDED task for control animals. Bar graph depicts the mean number of trials to criterion. Rats took significantly more trials to criterion to complete the extradimensional shift than the intradimensional shift (p = 0.005)*.

5.4.4. C-Fos expression:

As demonstrated in **figure 5.7,** there were no significant differences between the c-fos expression between ID and ED in the prelimbic cortex ($F_{1,6}$ = 0.015, p = 0.9), lateral orbital cortex ($F_{1,6}$ = 0.065, p = 0.8), medial orbital cortex ($F_{1,6}$ = 0.69, p = 0.4), ventral orbital cortex ($F_{1,6}$ = 0.52, p = 0.5), infralimbic cortex ($F_{1,6}$ = 0.001, p = 0.97), anterior cingulate cortex ($F_{1,6}$ = 1.07, p = 0.3) and the agranular insula ($F_{1,6}$ = 0.17, p = 0.69). These findings show no difference of c-fos expression in prefrontal areas between the ID- and ED-exposed

rats.

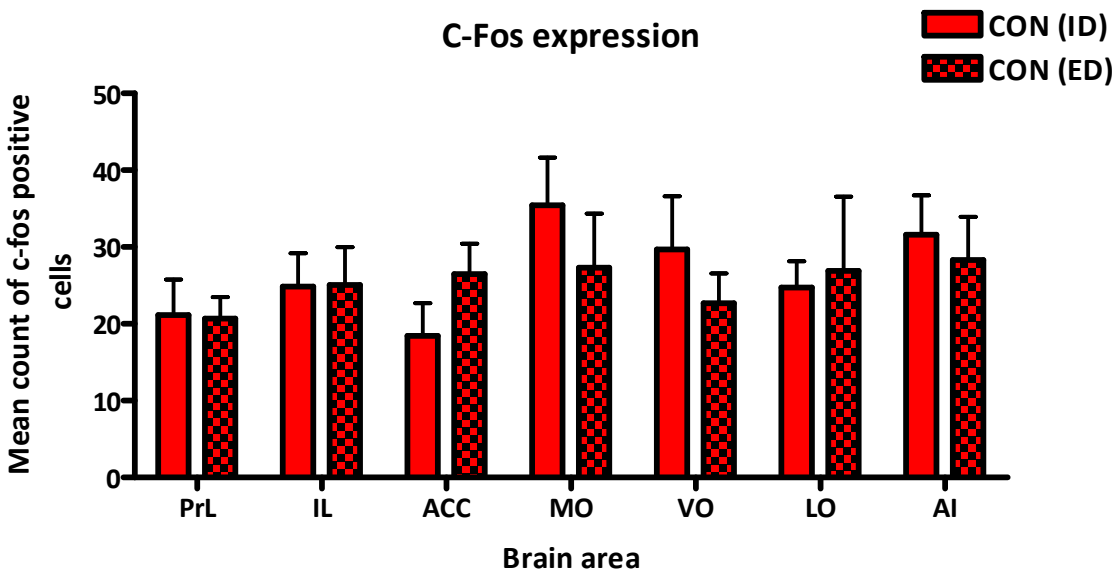


Fig 5.7 Mean ± SEM of c-Fos expression after exposure to the condensed version of ID or ED shift in control animals. Plain bars depict the rats that had undergone the intradimensional shift, checked bars show the rats that had undergone the extradimensional shift.

5.5. Discussion:

The main aim of the present study was to assess the effects of repeated ethanol withdrawal on attentional set shifting as a function of the prefrontal cortex using the IDED set shifting task and to investigate the PFC brain areas activated during both intradimensional and extradimensional shift in control rats.

The results from the first experiment of the present investigation indicated that repeated ethanol withdrawal did not significantly impairsuccessful performance in the IDED set shifting task. Although all rats required significantly more trials to reach criterion on the extradimensional shift, there was no significant difference across the groups. All rats performed the intradimensional shifts more rapidly than extradimensional shifts, which demonstrated that the rats had successfully formed a perceptual attentional set and were capable of shifting their attention to an extradimensional set (Birrell and Brown 2000).

In a lesion study by Birrell and Brown (Birrell and Brown 2000) using the IDED task, the authors found that lesions to the medial PFC, encompassing the prelimbic and

infralimbic cortices, resulted in a selective impairment in the ED shift in which lesioned rats took twice as many trials to criterion compared to control rats, indicating disrupted functioning of the medial prefrontal cortex. Lack of repeated ethanol withdrawal effects on IDED task would suggest no functional disruption of the medial prefrontal cortex. However, one could interpret these current findings in relation to the lesion study by Birrell and Brown (2000) as different approaches. Birrell and Brown's study (2000) investigated the function of the medial prefrontal cortex in the IDED task using lesions. This present study investigated the effects of repeated ethanol withdrawal on the medial prefrontal cortical function, a treatment which is not deemed to be as severe as brain lesions which are likely to produce more profound behavioural effects, more distinctly observed in rats compared to chronic ethanol treatment and withdrawal, which may produce more subtle behavioural effects. It is also possible that repeated ethanol withdrawal did not impair function in the medial prefrontal cortex. However, in a previous study of withdrawal-induced activation of the prefrontal cortex (see chapter 3), there were significantly higher levels of c-fos activation in the prelimbic cortex as a result of repeated ethanol withdrawal but no significant differences in c-fos activation in the infralimbic cortex compared to controls and rats which had experienced a single withdrawal episode. The medial prefrontal cortex comprises the prelimbic and the infralimbic cortices. Hence, from the previous c-fos data as reported in chapter 3, repeated ethanol withdrawal activated part of the medial prefrontal cortex responsible for successful attentional set shifting in the IDED task. It is possible that such a partial activation may not have resulted in a behavioural impairment as originally hypothesised. Furthermore, c-fos activation is a marker for neuronal activation but not necessarily of damage. Hence, c-fos activation may highlight the prefrontal brain areas sensitive to repeated ethanol withdrawal but these areas would not represent direct evidence towards prefrontal damage which would impair attentional set shifting ability. This finding contrasts with reports of heavy chronic alcohol dependence leading to frontal deficits in the WCST, amongst other cognitive tasks (Fitzhugh, Fitzhugh et al. 1960; Fitzhugh, Fitzhugh et al. 1965; Jones and Parsons 1971; Long and McLachlan 1974; Goldstein and Shelly 1980; Hill 1980; Parsons 1987; Parsons 1987; Sullivan 1993). However, these reports were found in human

alcoholics in which differences between the duration of alcohol abuse and the number of attempted detoxifications may have influenced experimental findings.

In the second experiment of the current study, the frontal brain areas involved in ID shift and ED shift were examined using c-fos expression as a measure of activation. There were no significant differences in c-fos expression between the control rats that had undergone the ID shift and control rats that had performed the ED shift. This current finding might suggest that an overlap exist in the prefrontal areas involved in both ID and ED shifts. The c-fos counts in control rats of this current experiment were comparable to the c-fos counts in control rats previously reported in chapter 3, indicating that there was agreement amongst studies in c-fos counts. Burnham and colleagues found increased Fos-like reactivity in the medial and orbital frontal cortices of rats performing extradimensional attentional shifts as well as reversals in the ED/ID task (Burnham, Bannerman et al. 2010). Although Burnham's study may be comparable to those of the present study in terms of experimental protocol, Burnham's study introduced a 90-minute break between the first four discriminations and the final discrimination of interest whereas in the present study, there was no break included for the experimental subjects hence this may have introduced complications in Fos expression detected in overlapping brain areas. Attributable to the acquisition of the initial discriminations.

The IDED task was devised as a rat analog of the Wisconsin Card Sorting Test, a neuropsychological test which is sensitive to damage to the frontal lobes. There is much evidence to show that prefrontal cortical damage impairs performance on attentional set shifting tasks in humans (Milner 1963; Nelson 1976; Stuss, Levine et al. 2000; Monchi, Petrides et al. 2001; Owen 2004; Monchi, Petrides et al. 2006). In a recent fMRI study on the WCST, the dorsolateral PFC was activated when feedback was provided in the task, indicating that the dorsolateral PFC plays a role in monitoring events in working memory (Petrides 1991; Petrides 1994; Petrides 2000; Ko, Monchi et al. 2008). The dorsolateral PFC in humans and primates has been found to be functionally homologous to the rat medial prefrontal cortex (Kolb 1990; Kesner 2000; Brown and Bowman 2002); hence the IDED task measuring attentional set shifting should activate the medial prefrontal cortex, when rats are required to shift

extradimensional set. Birrell and Brown (2000) conducted lesion studies to demonstrate that the medial PFC mediated attentional set shifting. The results from the present study indicate that extradimensional set shifting was not affected by ethanol treatment and withdrawal.

A possible interpretation of this finding may be that Birrell and Brown's (2000) lesion study produced profound behavioural impairments in attentional set shifting whereas the repeated ethanol withdrawal induced only mild or no impairments of the mPFC despite the c-fos induction. Furthermore, neuronal activation using c-fos as a marker will provide information about the sensitivity of brain areas activated during either ID or ED shift but does not represent an indicator of damage.

In summary, the results from this current study suggest that repeated ethanol withdrawal did not impair the functions of the prefrontal circuits on which attentional set shifting processes rely. No significant differences in brain activation were observed using c-fos expression to differentiate between intradimensional and extradimensional shifts, which may suggest that there was functional overlap of prefrontal brain areas involved in attentional set shifting. Alternatively, the c-fos method may be insufficiently sensitive to identify brain regions activated during the performance of these tasks.

Chapter 6

General Discussion

6.1: Summary of experimental findings

The results presented in this thesis serve to further our knowledge of the effects of repeated ethanol withdrawal on withdrawal severity as measured in neurobiological indices and behaviours relating to prefrontal cortical function. Repeated ethanol withdrawal, although leaving core body temperature and activity levels unaltered, results in a disruption of circadian rhythm and post-withdrawal water intake. However, the findings of disrupted circadian rhythm and enhanced water intake during the post-withdrawal period was not solely attributable to repeated ethanol withdrawal as singly withdrawn rats also demonstrated disruptions in circadian rhythm and greater effects on post-withdrawal water intake. Hence, these behavioural findings did not demonstrate greater withdrawal severity as a consequence of repeated ethanol withdrawal.

Repeated ethanol withdrawal produces a general increase in neuronal activation as revealed by c-fos expression in prefrontal cortical brain areas indicating the sensitivity of this brain region to repeated episodes of ethanol withdrawal, particularly the prelimbic cortex which also demonstrates increased neuronal plasticity using zif268 expression as a biological marker. This finding indicates, neurochemically at least, that repeated ethanol withdrawal results in PFC neuronal activation at a greater level than single withdrawal alone, which may predict disruptions to a wide range of behaviours mediated by the PFC, including response inhibition and attentional set shifting. However, it appears that in a novel 2-choice serial reaction time task devised in this laboratory to measure motor response inhibition, repeated ethanol withdrawal did not alter a rat's ability to inhibit a prepotent motor response (i.e. RWD rats showed similar impulsive responding profiles compared with CON and SWD rats). However, repeated ethanol withdrawal did result in attentional and learning deficits if learning occurred

after chronic ethanol administration. Learning deficits were not observed if learning occurred prior to chronic ethanol withdrawal.

Furthermore, repeated ethanol withdrawal did not impair attentional set shifting, a behaviour mediated by the medial PFC, indicating no further withdrawal severity on the medial PFC as a consequence of repeated ethanol withdrawal.

The findings of this thesis taken together indicate that repeated ethanol withdrawal produces neurochemical evidence of greater withdrawal severity. However, the implications of this neurochemical evidence did not translate into robust behavioural alterations measuring withdrawal severity. Aside from the current findings of ethanol withdrawal leading to disruptions to circadian rhythm and post-withdrawal water intake, repeated episodes of ethanol withdrawal did not produce further severity of behavioural dysfunction. Consistent with previous studies conducted in this laboratory (Stephens, Brown et al. 2001; Ripley, O'Shea et al. 2003; Ripley, Borlikova et al. 2004; Borlikova, Elbers et al. 2006), repeated ethanol withdrawal did result in more sessions to criterion compared to SWD and CON rats. Hence, it is possible that the increased neuronal activation of prefrontal areas may related to the learning deficits observed in RWD rats. Another possible interpretation may be that repeated ethanol withdrawal affects the brain regions associated with learning and memory located within the PFC.

Alcohol addiction, as with all drug addictions, is a chronically relapsing disorder which is characterised by compulsive drug taking and a loss of control in limiting drug intake. All drugs of abuse are known to act at both the nucleus accumbens and the ventral tegmental area (VTA), both brain areas that contain the mesolimbic dopamine pathway, which is a critical neural substrate of the reinforcing or addictive properties of all drugs of abuse. The mesolimbic dopamine pathway also extends to the amygdala, a structure of the brain’s limbic system that is linked to the processing of motivational significance of stimuli as well as mediation and control of major emotions (Breiter and Rosen 1999; Aggleton 2000; Pitkanen, Pikkarainen et al. 2000; Rolls 2000; Amaral, Capitanio et al. 2003; Everitt, Cardinal et al. 2003; LeDoux 2003).

Human studies conducted by Stephens et al (Stephens, Ripley et al. 2005) have reported binge drinkers show impaired fear conditioning. Impairments in fear

conditioning is related to dysfunction of the amygdala circuits (LeDoux 2003). Findings that repeated ethanol withdrawal leads to impaired fear learning in rats (Ripley, O'Shea et al. 2003) supports these data in human studies. Stephens and colleagues proposed that repeated ethanol withdrawal impaired the formation of associations between a tone stimulus and an aversive event which may also provide support for amygdala dysfunction as a consequence of repeated ethanol withdrawal (Stephens, Ripley et al. 2005). These results were supported by the finding that although CON and SWD rats showed high levels of c-fos expression in BLA and CeN after exposure to CS + tone predicting shock, RWD rats showed less c-fos expression indicating that repeated ethanol withdrawal impaired rats' ability to form associations between a tone and an aversive event. These findings from Stephens and colleagues may have wider implications for the treatment of alcoholics undergoing detoxification, who may show behavioural impairments in responding to aversive events such as punishment (Stephens, Ripley et al. 2005).

The role of the PFC in repeated ethanol withdrawal is less clear. The PFC is the seat of executive function and mediates cognitive control processes. Dysfunction of the PFC in human drug addicts has been linked to behaviours such as increased impulsive responding (Jentsch and Taylor 1999; Perry and Carroll 2008) and loss of response inhibition which may translate to a loss of control of drug taking or limiting alcohol intake.

6.2: Does repeated ethanol withdrawal lead to increased impulsivity?

Repeated ethanol withdrawal did not give rise to increased impulsive responding, as measured by the 2-CSRTT. Studies on alcohol-dependent patients have revealed deficits in motor, non-planning and attentional components of impulsivity (Salgado, Malloy-Diniz et al. 2009). However, these were measured in the period immediately after acute alcohol withdrawal whereas in the current thesis, impulsive responding was measured at 3 different time points in 3 separate experiments; namely 2-3 weeks after the final withdrawal, at which point any differences in impulsive responding may have dissipated or possibly all the animal subjects showed less than optimal performance due to the break in daily 2-CSRTT training sessions. Impulsivity was also

measured continuously throughout 2CSRTT training and probe trials, in which case no significant differences in impulsivity was observed between the SWD and RWD rats (chapter 4). It is possible that the continuous 2-CSRTT training led to optimal performance hence masking any differences in impulsive responding which may have been present. Another possible interpretation could be that the measures in Salgado et al's study (Salgado, Malloy-Diniz et al. 2009) measured commission errors in the continuous performance task, advantageous choices in the Iowa Gambling task and perseverative errors on the Wisconsin card sort test. These measures all represent different varieties of impulsivity and the measure more closely related to motor impulsivity in the 2-CSRTT is the commission errors in the continuous performance task. However, in the current thesis, repeated ethanol withdrawal did not result in increased motor impulsivity. A possible interpretation of this difference could be that although repeated ethanol withdrawal resulted in increased c-fos expression, repeated episodes of withdrawal did not induce sufficient damage to the prefrontal cortical brain regions to induce higher levels of impulsive responding. Furthermore, it is suggested that other brain areas aside from the prefrontal cortex play a role in motor impulsivity, such as the striatum and the basal ganglia (Besson, Belin et al.; Cardinal, Pennicott et al. 2001; Christakou, Robbins et al. 2001; Christakou, Robbins et al. 2004; Hariri, Brown et al. 2006; Bjork, Smith et al. 2008; Beck, Schlagenhauf et al. 2009). Borlikova et al (Borlikova, Le Merrer et al. 2006) reported that repeated ethanol withdrawal led to marked increases in c-fos expression in the nucleus accumbens, indicating that the basal ganglia showed greater sensitivity to this form of ethanol treatment. Nevertheless, increased sensitivity to repeated ethanol withdrawal did not give rise to increased motor impulsivity in the 2-CSRTT as reported in chapter 4. Motor impulsivity has been found to increase as a result of lesions to the anterior cingulate cortex (Muir et al 1996), infralimbic cortex (Chudasama et al 2003), and lesions that disconnect the mPFC from the anterior medial striatum (Christakou 2001). However, as the findings in chapter 3 show no significant differences in c-fos expression in the infralimbic cortex following repeated ethanol withdrawal compared with SWD and CON rats, this may explain the lack of effects of repeated ethanol withdrawal on motor impulsivity levels. Additionally, lesions studies conducted by Walton and Bannerman (2003) have found that large lesions to the medial PFC can substantially increase rats'

preference for small immediate reward over large delayed on the delay discounting task. However, in the current thesis, as no significant differences were observed in the IDED task which also tests medial PFC function, it is possible that repeated ethanol withdrawal may have little or no effect on impulsive choice either. Repeated ethanol withdrawal induced significantly higher c-fos expression in the anterior cingulate cortex compared to SWD and CON rats hence it was interesting to reveal the lack of differences in impulsive responding in the 2-CSRTT between the 3 treatment groups. From these collated findings, it appears that motor impulsivity may be affected by different PFC regions, and motor impulsivity may not be subject to mediation by a single PFC brain region. Furthermore, as human neuroimaging studies consistently report activation of the OFC in drug addicts (Volkow, Wang et al. 1999; Volkow and Fowler 2000; Volkow, Chang et al. 2001; Goldstein, Volkow et al. 2002; Bolla, Eldreth et al. 2003; Everitt, Hutcheson et al. 2007; Goldstein, Tomasi et al. 2007) it was interesting to note that despite c-fos expression of the OFC as a consequence of repeated ethanol withdrawal, this activation of the OFC did not translate to behavioural changes in rats with previous withdrawal experience. Additionally the OFC has been reported by McAlonan and Brown to mediate reversal learning in the IDED task in rats (McAlonan and Brown 2003); however, in the current thesis findings, repeated ethanol withdrawal did not impair reversal learning in IDED despite increased c-fos expression in this area following repeated ethanol withdrawal. This finding was consistent with the experimental reports of Borlikova et al (Borlikova, Elbers et al. 2006) in which repeated ethanol withdrawal did not impair spatial learning in the Barnes maze, a task which included an aspect of reversal learning. Thus, despite activation of the OFC, repeated ethanol withdrawal did not appear to impair reversal learning in the Barnes maze, however repeated ethanol withdrawal did impair reversal learning in a conditioned emotional response task, implicating dysfunction of amygdala circuits responsible for fear learning (Ripley, O'Shea et al. 2003).

Repeated ethanol withdrawal was found to result in learning deficits in the 2-CSRTT but only if learning occurred after the experience of repeated ethanol withdrawal, suggesting that repeated ethanol withdrawal impairs learning processes and disrupts

the ability to learn after repeated withdrawal experience. However, there was no impairment when learning occurred prior to the withdrawal experience.

6.3: Does repeated ethanol withdrawal impair attentional set shifting?

Attentional set shifting is one of the numerous functions of the prefrontal cortex (Robbins 2007). Two similar forms of cognitive flexibility, intradimensional shift and extradimensional shift are mediated by very different regions of the PFC (Dias, Robbins et al. 1996; Robbins 1996; Birrell and Brown 2000). Learning new reinforcement contingencies, which are switched to render a previously irrelevant dimension relevant, is comparable to the "category shift" in the Wisconsin Card Sort test because it involves shifting a response which requires the switching of attention between 2 perceptual dimensions, intradimensional and extradimensional shift. Repeated ethanol withdrawal did not lead to impairment in attentional set shifting in the IDED task, indicating that the integrity of the medial prefrontal cortex, responsible for mediating set shifting was not affected as a result of repeated episodes of ethanol withdrawal. This present finding stands in contrast to the results in human studies in which impairments in cognitive function as measured by neuropsychological tests including the WCST, were found in chronic alcoholics (Goldstein and Shelly 1980; Hill 1980; Parsons 1983; Parsons 1987; Sullivan 1993). Although in chapter 3, repeated ethanol withdrawal significantly increased c-fos expression in the prelimbic cortex, which is a prefrontal region within the medial PFC, it appears that partial activation of the medial prefrontal cortex did not sufficiently disrupt the ability to shift attentional set in the IDED task.

6.4: Does repeated ethanol withdrawal lead to greater withdrawal severity?

There was little evidence in the data collected in this thesis that rats which had undergone repeated episodes of ethanol withdrawal experienced more severe withdrawal symptoms as a consequence of a higher number of withdrawal experiences. Although at first glance, this finding does not appear to be consistent with the established literature in clinical and animal studies indicating greater withdrawal

severity is correlated with a greater number of withdrawal episodes, the data that support this view have been drawn from clinical studies measuring the number of seizures experienced by detoxified alcoholic patients (Ballenger and Post 1978; Brown, Anton et al. 1988) which was proposed by Ballenger and Post (1978) to be attributable to kindling-like brain mechanisms brought about by multiple ethanol withdrawals. Likewise, in animal models of repeated ethanol withdrawal conducted previously in this laboratory, there is evidence of kindling-like processes occurring as a result of multiple withdrawals (Maier and Pohorecky 1989; Becker and Hale 1993; Becker, Diaz-Granados et al. 1997; Stephens, Brown et al. 2001; Ripley, Dunworth et al. 2002; Ripley, Brown et al. 2003). However, these clinical and preclinical findings arise from seizure and kindling data, whereas the findings from the current thesis take behavioural measures of body temperature, activity levels, sleep architecture and post-withdrawal food and water intakes as measures for withdrawal severity. It is possible that withdrawal severity was more subtle in the measures employed in this current thesis and hence the differences could not be detected robustly. Another interpretation of the current findings could be that although repeated ethanol withdrawal produced greater withdrawal severity in seizures, it did not generalise to all behavioural measures in our rat model of repeated ethanol withdrawal. This view would be consistent with other measures such as anxiety in RWD rats using the same method of ethanol treatment.

However, as repeated ethanol withdrawal did not result in greater withdrawal severity, the current findings regarding greater levels of c-fos expression as a consequence of repeated ethanol withdrawal is particularly interesting as they indicate that brain activation during ethanol withdrawal increases with repeated withdrawal experience. Nevertheless, these data need to be interpreted with caution, as c-fos expression cannot be taken as a measure of damage to the prefrontal brain regions. However, higher levels of c-fos expression in the prefrontal cortex as an outcome of repeated ethanol withdrawal may be interpreted as greater sensitivity of the prefrontal cortex to repeated episodes of ethanol withdrawal which have not, in the findings of this current thesis, been found to translate to behavioural changes nor does increased sensitivity generalise to all the behavioural measures investigated.

6.5: Limitations

A primary limitation of the work undertaken in this thesis is that in our rat model of repeated ethanol withdrawal, the magnitude of withdrawal-related effects on behavioural indices is determined the animals' ethanol consumption level. In 2 studies in this thesis, (see chapter 2, page 66 and chapter 4, page 135), chronic ethanol consumption was considerably lower compared with other studies conducted in this thesis as well as previous experiments in the laboratory. Therefore, within a treatment group there may be differences in behaviour which may be attributed not to the withdrawal experience but to the level of ethanol consumption. Although difficulty in maintaining stable chronic ethanol consumption is a constraint of the rat model used, this method of administration has produced behavioural deficits in rats which are strikingly consistent with findings in human alcoholics.

In addition, the EEG/EMG study (chapter 2) conducted in the current thesis resulted in a disruption of circadian rhythm in control animals, in which control rats showed higher activity levels during the light phase compared with lower activity levels in the dark phase. It is well established that rats exhibit higher activity levels throughout the dark phase and as previously discussed in chapter 2 (page 74) this circadian rhythm disruption may possibly have been attributable to the liquid diet administration regime. However, this is a possible interpretation of the findings and confirmation of this interpretation would required further investigation.

The 2-CSRTT was devised to measure prepotent response inhibition / impulsive responding and was modelled on the well established 5-CSRTT. However, it must be noted that differences between the instrumental responses of lever pressing as employed in the 2-CSRTT and of nose poking in the 5-CSRTT may have influenced the ease by which impulsive responding in measured. Nose poking appears to encourage higher levels of impulsive responding in comparison with lever pressing hence subtle differences in impulsive responding as a consequence of repeated ethanol withdrawal may not have been detected using the methods of the 2-CSRTT.

Additionally, all experiments in this thesis were conducted using male rats. Hence, any sex differences related to repeated ethanol withdrawal would have been excluded.

There is evidence in human studies indicating that females are more susceptible to the harmful effects of alcohol and alcohol withdrawal (Townshend and Duka 2005) although several studies have postulated that female sensitivity to alcohol may be generalised to all drugs of abuse not just alcohol (Carroll and Anker; Lynch, Roth et al. 2002; Carroll, Lynch et al. 2004; Roth, Cosgrove et al. 2004). Furthermore, in rodent studies ethanol intake was found to be higher in females than in males (Finn, Beckley et al.; Belknap, Crabbe et al. 1993; Finn, Sinnott et al. 2004; Chester, Barrenha et al. 2008). The reason for this increased effect in female rats is possibly because they are smaller than male rats and therefore achieve a higher blood ethanol concentrations compared with male rats. Therefore, the inclusion of female rats in these experiments could provide a more complete analysis of the effects of repeated ethanol withdrawal.

6.6: Further Work

In order to test the question of increased impulsivity as a result of repeated ethanol withdrawal, it would be useful to test a different variety of impulsivity, i.e. impulsive choice, using the delayed discounting task to investigate whether findings would bridge the gap between the human data reporting increased impulsivity as a consequence of ethanol withdrawal and to assess these findings in relation to our rat model of repeated ethanol withdrawal. Increased c-fos expression in the prefrontal cortical areas may relate to functional and behavioural changes and, as proposed experiments may involve investigating the effects of repeated ethanol withdrawal on impulsive choice using the delay discounting paradigm. Furthermore, it would be interesting to assess the effects of repeated ethanol withdrawal in rats on the established five choice serial reaction time task in order to assess whether results would be comparable with the reported findings from the 2-CSRTT in this current thesis.

It would be useful for further work to be conducted into investigating the actual neuronal cell death in the prefrontal cortex using methods such as amino cupric silver staining following repeated episodes of ethanol withdrawal in order to compare findings with single withdrawal and control animals. This measurement would provide

a more reliable measure of neuronal cell death and may provide support for similar areas activated by repeated ethanol withdrawal as detected using c-fos expression.

6.7: Conclusions

Our previous studies in the laboratory concerning repeated ethanol withdrawal have consistently reported learning deficits in rat subjects that had previous experience of withdrawal. In this thesis, repeated ethanol withdrawal led to learning deficits when ethanol withdrawal occurred prior to learning, implicating that repeated ethanol withdrawal leads to disruptions of learning systems within the brain but did not impair learning already acquired prior to the withdrawal process. However, it is interesting to note that in previous studies in this laboratory, all the behavioural deficits reported have consisted of Pavlovian associations and Pavlovian conditioning, whereas the behaviours investigated in this current thesis have involved more cognitive abilities, requiring maintaining and shifting attentional set (IDED task) and response inhibition (2-CSRTT). It may thus be arguable that repeated ethanol withdrawal did not disrupt higher cognitive processes mediated by the prefrontal cortex, which may explain the lack of effect of chronic ethanol consumption and withdrawal on the measures of PFC function investigated in this thesis.

Chronic ethanol consumption and withdrawal led to disruption of circadian rhythm; however, this effect was not exacerbated by repeated episodes of ethanol withdrawal. However, there is evidence indicating a “toughening up” effect resulting from multiple withdrawals as seen in previous studies conducted in this laboratory involving spatial learning, contextual fear conditioning, and anxiety and depressed LTP in the amygdala and hippocampus, both limbic brain areas which are heavily implicated in fear processing, learning and memory systems (Ripley, O'Shea et al. 2003; Stephens, Ripley et al. 2005; Borlikova, Elbers et al. 2006; Borlikova, Le Merrer et al. 2006).

Repeated ethanol withdrawal did not result in increased motor impulsivity although other measures of different varieties of impulsivity were not explored in this current thesis. Additionally repeated ethanol withdrawal did not lead to increased severity of withdrawal symptoms as measured by sleep architecture, core body temperature, activity levels or post-withdrawal food intake. Repeated ethanol withdrawal produced

significantly increased levels of c-fos expression in prefrontal cortical areas including the prelimbic cortex; however, partial activation of the medial prefrontal cortex did not result in impairments in attentional set shifting.

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