A behavioural and electrophysiological study of factors involved in the relationship between stress and alcohol dependence

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Abstract

Alcohol dependence causes disruption to both work and family life and the associated costs are £150+ million in the UK alone. Stressful life events play a role in initiation of uncontrolled (dependent) drinking and can precipitate relapse to high ethanol consumption after treatment / abstinence.

The primary neurological substrate for ethanol reward is the mesolimbic dopamine system of the medial forebrain bundle. Activation of the hypothalamopituitaryadrenal axis (the hormonal response to many stresssors) plays a role in the control of ethanol consumption and relapse, and modulation of neuronal activity by chronic calcium channel blockade decreases ethanol intake, tolerance and withdrawal. The stress system and calcium channel blockade both affect the dopaminergic reward pathways.

Hypothesis: Stress and the stress hormone, corticosterone, play a crucial role in the modulation of ethanol consumption and the long term changes resulting from chronic ethanol intake.

This hypothesis was tested by investigating the effects of:

- social status and calcium channel blockade on chronic ethanol intake (free choice 5, 10, 20% ethanol and water) of group housed rats.

- social stress from defeat by an aggressive resident on ethanol preference of low ethanol preference C57 mice.

- 6 days abstinence from chronic ethanol intake (liquid diet) on NMDA-stimulated firing of dopaminergic, ventral tegmental area, cells and the role of corticosterone in modulation of this response to NMDA.

The main findings from these studies indicate that, while the social stress of group housing under laboratory conditions may be insufficient to elevate ethanol intake, repeated defeat significantly increases ethanol intake. However, neither chronic ethanol consumption nor corticosterone seemd to have any effect on NMDA-stimulated dopamine cell firing.

These results indicate a significant role for social stress in the modulation of ethanol intake but possibly not via the action of corticosterone on NMDA-stimulation of the mesolimbic dopamine system.

A behavioural and electrophysiological study of factors involved in the relationship between stress and alcohol dependence.

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Dedicated to my wife, Kim.

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None of the work contained in this thesis has previously been submitted for a degree in this or any other University.
No material has been generated from joint work, and material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

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Abbreviations

ADE	Alcohol deprivation effect
AMPA	DL-α-3-hydroxy-5-methylisoxazole-4-propionate
GABA	γ-amino butyric acid
HPA axis	Hypothalamo-pituitary adrenal axis
i.p.	Intra-peritoneal
NMDA	N-methyl-D-aspartic acid
VTA	Ventral tegmental area

Chapter 1

Introduction

General Introduction

Ethanol and its actions

Ethanol (ethyl alcohol - C_2H_5OH) is a small molecule (MW 46.07) with a high water solubility due to its size and the dipole moment induced by the ethyl group at one 'end' of the molecule and the hydroxy group at the other 'end'. However, ethanol also has a relatively high oil/water partition coefficient of 0.03 (eg., compared to 0.00003 for glucose and 0.00015 for urea). These distinct physical characteristics mean that ethanol is readily carried around the body by the blood stream and quickly diffuses into all tissues or organs. Yet at the same time, the blood brain barrier offers no real resistance to the diffusion of ethanol into the cerebrospinal fluid and brain tissues, and ethanol can also readily diffuse into the lipid bilayer of the cell surface membrane and through to the intracellular domain.

The speed of ethanol delivery to all body systems, particularly the brain, is such that mildly intoxicating feelings can be experienced within minutes of drinking a relatively small volume of ethanol, and the ethanol naïve body and brain is highly sensitive to very low levels of ethanol (blood alcohol levels below 5mM or 22mg%; Little, 1999).

In the case of alcohol dependence, however, individuals drink far higher levels of ethanol at a far faster rate than the 'average' social drinker. An alcoholic patient can (and does) consume several fold higher levels of ethanol (usually several bottles of spirits in a given session) with fewer of the subjective effects reported by naïve drinkers or 'social' drinkers and an 'average' alcoholic patient will consume sufficient in any given session to kill an ethanol naïve individual (Robin *et al.*, 1998).

Acute or short-term administration of ethanol has a multitude of effects on the normal functioning of physiological processes in both the central nervous system and peripheral systems. Acute ethanol administration is generally considered to be

excitatory in its initial phase and inhibitory at higher doses or with slightly longer exposure. Table 1.1 (from Little, 1999) shows the relevant blood (plasma) ethanol concentrations to produce symptoms ranging from mood alterations (usually uplifting initially, although depressant mood effects are present at higher concentrations or with longer exposure) to full intoxication, loss of consciousness and death. A detailed discussion of the relevance of ethanol dose in research applications is presented later (page 15).

Table 1.1
Plasma ethanol concentrations at which the behavioural effects of ethanol have been reported in humans and laboratory rodents (Little, 1999).

Effect	mM	mg%
Humans		
Alterations in mood	5	22
Impaired attention; increased accident risk	11	50
Ataxia	20	90
Intoxication	44	200
Loss of consciousness	50	227
Death	111	500
Rodents		
Sedation	22	100
Ataxia	27	122
Loss of righting reflex	66	300

Following prolonged drinking (or repeated administration of ethanol) the effects of acute ethanol on neurotransmitter release, protein function and expression (including neurotransmitter receptors and ion channels) compound to such a degree as to result in depressed activity and / or sedation. Behavioural changes such as decreased activity and depressed mood, loss of motor control and eventually unconsciousness are thought to result primarily from the activation of inhibitory γ-amino butyric acid (GABA) function (Mehta and Ticku, 1999), and inhibition of the excitatory glutamatergic system (Dodd *et al.*, 2000).

The depressed central function induced by chronic or repeated ethanol exposure is combated by homeostatic adjustments in receptor sensitivity, expression level, and functional coupling as well as physiological adaptations in the neuronal

networks in order to override ethanol-induced depression by increasing excitatory signals and decreasing inhibitory signals. In overview, the inhibitory GABAergic system is down-regulated, whilst the sensitivity of the NMDA receptor-mediated glutamatergic system is increased, resulting in decreased inhibitory function and increased excitatory activity. In conjunction with increased voltage sensitive calcium channel density, these changes result in increased neuronal excitability to counter-balance the inhibitory effects of ethanol (for a review see Finn and Crabbe, 1997). These changes eventually return the overall functionality of the central nervous system to much the same levels as before the exposure to repeated ethanol, ie the development of functional tolerance to ethanol (Figure 1.1).

The development of physiological or functional tolerance to the disruptive effects of ethanol is not instantly reversed on the cessation of ethanol intake (withdrawal), and so the compensatory mechanisms working to equilibrate the effects of the continued presence of ethanol are still in place during the withdrawal period, resulting in an overshoot of these systems and behavioural perturbations that are described as the 'physical withdrawal syndrome'. This balancing act between the effects of ethanol and the body's adjustment to these effects is the essence of physical dependence on ethanol (or other drugs of abuse and addiction) as described by Goldstein (1971). This 'homeostat hypothesis' has provided a firm groundwork upon which a lot of research into alcohol dependence and withdrawal has been successfully carried out. However, the idea is perhaps an oversimplification of the long term effects of chronic ethanol intake as it overlooks the effects of ethanol that are maintained for several weeks after the cessation of ethanol activity. If physical dependence was simply a homeostatic change then these mechanisms should (in theory) eventually reset themselves once the ethanol was removed. This is true for many of the ethanol induced changes that cause the physical withdrawal syndrome which lasts for 24-48 hours maximally (Goldstein, 1971). However, there are far

longer lived effects of ethanol that last beyond the end of the physical withdrawal syndrome. For example, Bailey *et al* (2000) demonstrated changes in dopamine metabolism 2 months after withdrawal from ethanol liquid diet; Manley and Little (1997) demonstrated increased locomotor sensitivity to amphetamine and cocaine 6 days and 2 months after withdrawal from ethanol liquid diet. The longevity of these changes implies that they are not produced by an overshoot in regulatory mechanisms but a more permanent modulation of certain central systems by chronic ethanol.

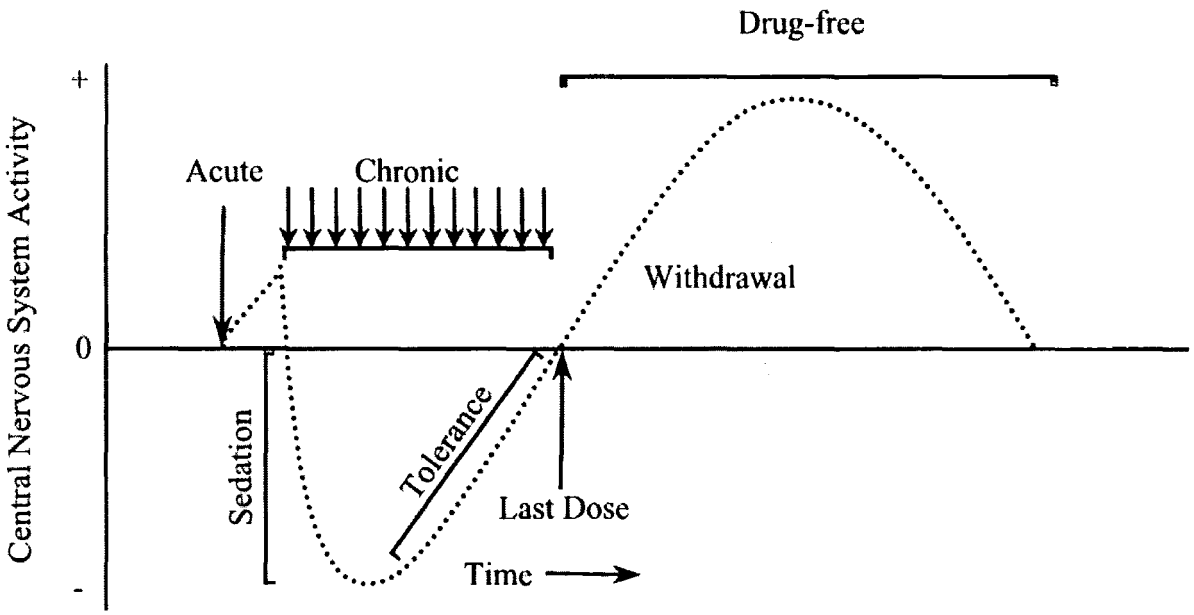


Figure 1.1: Graphical representation of central nervous system activity during the various phases of the development of ethanol addiction (adapted from Finn and Crabbe, 1997).

Human alcohol dependence

The social and economic costs of alcohol abuse and dependence

Alcohol dependence is one of the most expensive disorders of the western world in terms of lost earnings, health care costs and effects on society. The healthcare charity, Alcohol Concern (www.alcholconcern.org.uk – fact sheet 16), lists the UK National Health Service expenditure as approximately £150 million a year on alcohol-related health problems, while alcohol related crime costs around £50 million and the cost to industry through lost productivity and workforce replacement is estimated at £2 billion per year. The statistics listed on the UK Department of Health

web site (www.doh.gov.uk) estimate the incidence of alcohol dependence in private households as 75 per thousand people for men between ages 16 and 64 and 21 per thousand people in women of the same age range.

The most recent statistics provided by the National Institute on Alcohol Abuse and Alcoholism (www.nida.nih.gov) in the United States of America estimated costs for 1995 totalling $276,500 million (~£191,000 million) for drug dependence, of which $166,500 million (~£119,500 million) was from alcohol dependence. These estimated costs break down to: $24,000 million for 'health care' costs; $24,700 million for the 'effects on society' such as crime, social welfare administration; $119,250 million in 'lost earnings' such as institutionalisation, premature death, impaired productivity. The latter of these costs is a predicted deficit rather than an outlay required to treat the disorder, but still a significant driving force for the treatment of alcoholism in the USA.

In addition to the large costs of the treatment of ethanol dependence, a very large proportion of alcoholic patients relapse to dependent alcohol consumption within the first year of abstinence and very little is understood of the mechanism of action of ethanol, and so there are few truly effective drugs available specifically for the treatment of alcohol dependence (Miller *et al.*, 2001; Sinclair, 2001).

Laboratory Models of Alcohol Dependence

The requirements for a model of alcohol dependence

The term alcohol dependence is used to describe a syndrome with multiple characteristics. Diagnosis of ethanol dependence is based around adaptations of seven "essential elements" described by Edwards and Gross in 1976:

1. subjective awareness of a compulsion to drink;
2. narrowing of the drinking repertoire;
3. increasing priority and prevalence of drink-seeking behaviour;
4. increased tolerance to alcohol (often resulting in increased consumption);
5. repeated withdrawal symptoms;
6. relief or avoidance of withdrawal symptoms by further drinking;
7. reinstatement of drinking (at similar levels) after abstinence.

These seven characteristics are integral to the currently used diagnostic criteria listed by both the World Health Organisation (ICD-10, 1992) and the American Psychiatric Association (DSM-IV; 1994) for alcohol dependence (Appendix page 214). However, none of the rodent models of ethanol dependence available for use in the laboratory meets more than one of these criteria (Wolffgramm and Heyne, 1995).

In order to study effectively the mechanisms involved in alcohol addiction and the high level of ethanol consumption that goes along with this we need to be able to model the human disorder in terms of both the transition from occasional or 'social' drinking to dependence as well as the physical aspects of withdrawal and the subsequent relapse to drinking (in a dependent manner) following periods of abstinence. The first step would seem to be to provide ethanol in a form that is ingested and metabolised as normally as possible since this is how a human alcoholic

receives their ethanol, and the metabolic by products may play as important a role in the effects of ethanol as ethanol itself does. There are several animal models of ethanol consumption in regular use, indeed many have been used for several decades. These models can be split into two general categories:

1) Forced ethanol consumption: individuals are provided ethanol in conjunction with another vital nutrient so that the ethanol intake levels are high (usually in excess of 10g/kg/24h) and there is little inter-subject variability except for that due to the differing caloric requirements of each individual. One method of forced ethanol intake that is less commonly applied today, is the continuous forced inhalation of ethanol vapour. This method provides a continuous high level of ethanol intake by housing animals in a closed chamber containing vaporised ethanol (8-15 mg / l of air; Goldstein, 1972). This technique has been used very successfully for the investigation of the physical withdrawal syndrome following high levels of ethanol intake, (for example see Goldstein and Pal, 1971). However, ethanol exposure is at a continuous level controlled by the respiration rate of each individual and does not follow the more common intermittent pattern of ethanol intake observed in human alcoholics (Tomsovic *et al.*, 1974).

Ethanol can be provided as the sole drinking fluid (diluted with water to a concentration that the animals will consume – typically to between 10 and 20% v/v). Whenever the individual drinks due to water needs, they also consume ethanol. This provides a consistent level of intake paralleling the test animals circadian rhythm of fluid requirements. Control animals receive only water as their drinking fluid. However, the ethanol consumed in this manner provides a significant number of calories which the control animals do not receive as well as providing taste stimulation (or an aversive taste depending on the ethanol concentration provided). These factors mean that, while this method works well at producing animals with

experience of high levels of ethanol intake, the parallel controls have not received the same environmental stimuli of taste, nor the same nutritional inputs. Thus this model of ethanol intake is perhaps not the best method for producing a well balanced group of test and control animals to investigate the effects of high levels of ethanol intake.

Provision of dilute ethanol as the sole drinking fluid does have distinct uses as an easy to apply method of 'preparing' animals for exposure to ethanol via other methods of administration. For example, Janak *et al.*(1998) used a 3 day period of access to 10% ethanol in tap water prior to training Long-Evans rats to respond for ethanol in an operant self-administration model. The 3 days of ethanol access allows the animals to "get a taste" for alcohol prior to the operant training with mixtures of sucrose and ethanol solutions.

A third method of forced ethanol consumption is the provision of ethanol and all nutritional calories as part of a liquid diet. This technique has been refined over years of use and now several recipes are available, the most common being the diets of Frye *et al.* (1981) and Lieber and De Carli (1973). These diets provide all vitamins, minerals and calories in a single liquid form and are calorie controlled such that the calories provided by the ethanol in the 'ethanol diet' is balanced by the addition of extra sugar calories in the 'control diet'. These two diets are very commonly used across the field of alcohol research, although it is possible to achieve much the same effect using a total nutrition diet drink such as the Ensure or Slimfast milkshakes. Indeed many laboratories use these diets, rather than the more expensive diets of Lieber and De Carli (1973) or Frye *et al.* (1981) with great success. However, I would question whether a dietary system designed for humans trying to regulate their caloric intake provides adequate nutrition for rodents and whether the nutrient provision is as good as that of a diet specifically designed to provide the essential amino acids, vitamins and minerals required by laboratory rodents.

Ethanol containing liquid diets are very successful at providing animals with high levels of ethanol intake (around 25-30 g/kg/24h was achieved in the work presented here). In addition to the very high levels of ethanol intake achieved, control animals receive a closely calorie matched diet. Ethanol intake is not continuous, but rather follows the circadian feeding rhythms of the animals and so should be a closer parallel to the intermittent peaks of ethanol intake seen with human alcoholics. One suggested problem with the provision of all nutrition as a liquid diet, however, is that the animals receive no solid food to chew. The chewing of hard food (such as standard laboratory chow) gradually wears down the constantly growing teeth of rodents, thus a protracted period with nothing to chew can result in rats with longer than normal front teeth. However, while this is not a normal situation for the animal it does not seem to present huge difficulties unless liquid diets are provided for very long periods (months rather than weeks).

Forced ethanol consumption produces animals that have consumed a high level of ethanol over a controllable length of time. However, voluntary consumption of ethanol (despite negative physiological, psychological and social effects) is a crucial aspect of ethanol dependence in humans and in these models of forced intake animals have no real control over their ethanol intake. Interestingly, however, increased tolerance to ethanol is produced by these methods and (other than inhalational administration) the range of drinking fluid type is limited potentially mimicking the narrowed drinking repertoire that is characteristic of human ethanol dependence (Edwards and Gross, 1976).

2) Free choice access to ethanol: individuals are allowed open access to an ethanol containing solution at the same time as water is present as their primary drinking fluids. The most classical method of access is the preference model in which animals have a free-choice between dilute ethanol (usually 6, 8 or 10% but sometimes

as high as 20% v/v) and water. Daily measurements of ethanol and water consumption can be made from this method and, to some degree, factors affecting the desire for ethanol can be investigated. This method provides a good model of voluntary consumption of ethanol as a chosen drinking fluid however, while it is possible for the animals to consume more and more ethanol with increasing exposure, these animals generally do not show the escalating uncontrolled ethanol consumption or ethanol seeking behaviour characteristic of human ethanol dependence, nor is the absolute level of ethanol consumption usually higher than 2-3 g/kg/24h (Wolffgramm and Heyne, 1995).

A more advanced measure of the drive to receive ethanol, however, uses operant responding for ethanol. In this method animals are trained to press a lever (or poke their nose in a hole) in order to receive a small volume of ethanol. Operant self-administration of ethanol provides a robust yet flexible method of investigating the reinforcing properties of ethanol, i.e. the balance point between 'beneficial' pharmacological effects and the amount of work required to obtain ethanol. Potent reinforcers will maintain a high level of lever pressing for low doses of drug. Through manipulations of various aspects of this technique such as the time between ethanol presentations (Beardsley *et al.*, 1983), the number of lever presses required per ethanol presentation (Meisch and Thompson, 1973; Grant and Samson, 1985) and the extinction of responding by the provision of water not ethanol on successful lever pressing (Janak *et al.*,1999; Le *et al.*, 1999, 2000). This technique allows for relatively sophisticated measurement of the drive to drink ethanol and the factors involved in the reinforcing and rewarding properties of ethanol. However, the average daily ethanol intake is usually less than 3 g/kg (Grant and Samson, 1985) and access to ethanol is commonly limited to one test session per day lasting 20 to 30 minutes (Grant and Samson, 1985; Janak *et al.*., 1999; Le *et al.*, 1999, 2000). This method of ethanol administration is thus a good measure of ethanol reward and drug

seeking behaviour but lacks several aspects of human alcohol dependence: continuous, cyclical ethanol intake (Tomsovic, 1974); a high level of uncontrolled ethanol intake (Wolffgramm and Heyne, 1995); and any overt physical withdrawal syndrome (Beardsley *et al.*, 1983; Grant and Samson, 1985; Janak *et al.*, 1999; Le *et al.*, 1999, 2000).

The ethanol withdrawal syndrome

The human withdrawal syndrome

The human ethanol withdrawal syndrome is thought to be a crucial factor in the maintenance of drinking behaviour and encompasses a wide range of symptoms Twenty eight symptoms are listed across the 18 diagnostic screens currently in clinical use (reviewed by Williams *et al.*, 2001) (Table 1.2), although no single screen looks for all of these symptoms.

Animal models of the physical withdrawal syndrome

One of the largest areas of research into alcohol dependence over the last 15-20 years has been that of the physical withdrawal syndrome resulting from termination of ethanol access after long term, high levels of intake. The human withdrawal syndrome (as outlined above) is highly reproducible in rodents. Characteristic behavioural symptoms were originally described by Freund (1969) and Goldstein (1971) and the range of withdrawal reactions for each symptom are shown in Table 1.3.

Table 1.2 Symptoms of the physical ethanol withdrawal syndrome	
Anxiety	Headache
Restlessness	Tremor
Irritability	Impaired co-ordination
Depression	Altered consciousness
Anorexia	Concentration deficits
Nausea	Dizziness
Vomiting	Neurological
GI Disturbance	Insomnia
Temperature	Sleep Disturbances
Sweating	Hallucination
Flushing	Visual disturbances
Tachycardia	Auditory disturbance
Palpitations	Tactile disturbance
Hypertension	Delusions

Table 1.3: The main characteristic symptoms of the rodent ethanol physical withdrawal syndrome (from Goldstein, 1971).

Sign	Minimum degree	Maximum degree
Lethargy	Slow movement, dragging limbs	Coma
Tremor	Twitching, occasional trembling	Continuous tremor
Tail lift	One per hour	Four per hour
Startle to noise	Twitch	Convulsion
Convulsion to handling	Mild, tonic	Severe, clonic
Spontaneous convulsion	Mild, tonic; jumping	Three or more generalised tonic-clonic convulsions per hour
Death		Death

In addition to the overt behavioural symptoms of withdrawal, more subtle physiological changes occur on withdrawal from chronic ethanol. Core body temperature drops and heart rate increases on repeated withdrawal from chronic ethanol, even at levels of ethanol intake that do not elicit the behavioural signs of the physical withdrawal syndrome (3-5 g/kg/24h) (Holter *et al.*, 2000). Hyperexcitability

has been reported for multiple brain functions including, but not limited to, visually evoked potentials recorded in the visual cortex, reticular formation, and thalamus (Begleiter and Porjesz, 1977); and stimulated hippocampal activity measured at the whole animal level (induction of forelimb clonus; Geisler *et al.*, 1978) and at the cellular level (orthodromically stimulated hippocampal (CA1) cell firing; Whittington and Little, 1991).

While the withdrawal syndrome has been well classified, little research has been done beyond the first 24 hours after cessation of chronic ethanol intake. Sinclair and Senter (1967) described an increased ethanol intake level following withdrawal and reintroduction to ethanol after a period of abstinence. This 'alcohol deprivation effect' reported by Sinclair and Senter (1967) has been further manipulated by Spanagel *et al.* (1996) and Holter *et al.* (2000) to elevate the 'baseline' levels of ethanol consumption of Wistar rats by exposing them to 3 day periods of abstinence once a month. Both sets of authors reported a successful elevation of intake from ~3 g/kg/24h in animals with uninterrupted ethanol access to ~5 g/kg/24h in animals exposed to repeated abstinence periods.

Despite the clear methodological advantages of high intake rats with minimal manipulations, such as in the studies of Holter *et al.*, (2000) and Spanagel *et al.*, (1996), and the obvious parallel between these studies and the repeated periods of withdrawal (and detoxification) experienced by most alcoholics (either deliberately or due to interruptions in their supplies of alcohol and/or money), historically there has been little therapy based research into the alcohol deprivation effect.

More recently the return to drinking after a period of abstinence has been seen as a potential therapeutic target, and an important focus for basic and clinical research. The characteristic of relapse to ethanol drinking that seems to be most important is that the intake level after abstinence returns immediately to the high levels experienced during periods of established drinking. This is equally true for

human alcoholics who remain tolerant to ethanol despite abstinence and will relapse following a large 'binge' rather than gradually increasing their drinking as they did when they originally acquired the addiction (Robin *et al.*, 1998).

In light of the long term changes in mesolimbic dopamine function discussed on page 19 and the large number of abstinent alcoholics that relapse within the first year of abstinence (Robin *et al.*, 1998), the changes induced by chronic ethanol that remain several hours or days after withdrawal from ethanol is a vital area for the understanding of ethanol's actions and the development of treatment strategies and drug therapy.

Many of the models of ethanol consumption and withdrawal in current use share only a very limited number of characteristics with human alcohol dependence and this remains a major weakness of whole animal modelling of ethanol consumption and the factors modulating ethanol dependence. As well as matching the criteria used for diagnosis of ethanol dependence in whole animal models, cellular assays of ethanol's actions also need to have relevance to the human condition. The primary issue of importance in studies of the *in vitro* effects of ethanol is the use of ethanol concentrations that are similar to those seen under normal and alcoholic drinking conditions.

Ethanol concentrations in brain and plasma and their relevance to behavioural effects of ethanol.

In order to study the effects of ethanol in experimental systems that are highly controlled and easily manipulated, one needs to consider the ethanol concentrations that are relevant to physiological levels of ethanol attainable in human alcoholic subjects. This point is especially pertinent to *in vitro* studies where, often, such high levels of ethanol are used as to be fatal to an intact animal. Although similarly high levels can be produced through ethanol injections the problem does not arise quite so

obviously with *in vivo* applications of ethanol (the obvious exception being direct, focal application to brain regions via dialysis probes or iontophoresis). Table 1.1 (page 3) shows the behavioural effects of various plasma concentrations of ethanol.

Plasma ethanol concentrations in the region of 5-20mM are generally perceived as intoxicating levels of ethanol resulting in mood changes, anxiolysis, excitation and impaired cognition, while higher plasma levels between 20 and 50mM are associated with sedation and impaired motor co-ordination. Ethanol concentrations higher than 50-100mM are consistent with anaesthesia and a distinct risk of fatality (Little, 1999). In 1957, Kaye and Haag described a high correlation between alcohol poisoning resulting in death and blood alcohol levels equivalent to between 39 and 130mM. However, Nurmi *et al.* (1996) demonstrated that brain ethanol levels elevated faster than plasma levels (within 5 minutes of i.p injection) and then levelled off to the same as plasma levels after 20 minutes. Thus plasma levels of ethanol are partially indicative of the levels of ethanol found in the brain after the first 20 – 30 minutes of ethanol exposure. The initial peak in ethanol levels immediately after ethanol administration is distinctly higher in the central nervous system and is not easily predicted by the circulating plasma levels of ethanol.

Many of the studies discussed in this thesis have used concentrations of ethanol far exceeding the concentrations causing anaesthesia and death. This is most common in *in vitro* studies of the electrophysiological effects of ethanol on brain slices or single cells. Deitrich and Harris (1996) discussed the validity of these measurements in terms of ethanol as a pharmacological agent used to investigate physiological systems. The higher levels of ethanol used are not a good representation of a physiological condition and, since ethanol is essentially a 'dirty' drug with a multitude of sites of action that are not all clearly understood or defined, it seems that as a pharmacological tool it is far from ideal. In fact, I would suggest that the 'ethanol as a pharmacological agent' idea is a *post hoc* justification for

unreasonably high ethanol concentrations being used without any regard for physiological relevance.

Another, more reasonable, justification for the *a priori* decision to use high concentrations of ethanol (fatal or anaesthetic levels) is to confirm that the stimulatory dose response curve is sigmoidal in shape and not bell-shaped. In other words, to check whether very high concentrations of ethanol are depressant on the system being examined, which might implicate the system tested as significant in the anaesthetic (and/or fatal) actions of high ethanol concentrations.

It seems that lower concentrations of ethanol, whilst producing significant behavioural effects, are not generally considered experimentally useful because of the subtlety of their effects. Indeed, several authors have discussed ethanol concentrations high enough to cause anaesthesia and death as 'low concentrations' of ethanol when discussing them within the context of the pharmacological effect they were examining and have ignored the true relevance of these concentrations in intake animals and humans (eg, Yim, *et al.*, 1998). However, studies using the application of ethanol via the microdialysis probe (eg, Yim, *et al.*, 1998; Yan *et al.*, 1996) state that they are using very high concentrations of ethanol (0.16-1.6M in the latter case) yet, since the ethanol is applied by (albeit rapid) diffusion across the dialysis membrane and into the tissues, the actual concentration present in the brain is not clear and may not be anywhere near as high as they have quoted.

The effects of ethanol on neurotransmitter systems and neuronal activity.

The central actions of ethanol appear to be mediated through many direct and indirect mechanisms (Hunt, 1996; Lovinger, 1997; Kallmen and Gustafson, 1998; Faingold *et al.*, 1998). However, the two key mechanisms of relevance in the maintenance of ethanol intake, in terms of ethanol abuse and dependence, are the activation of the reward system and reinforcement of behaviours related to drug seeking and administration (Wise 1998).

Historically, reward was considered to be the positive feelings resulting from a particular action or activity such as consumption of food or drugs, or sexual intercourse and this definition still holds true for the modern use of the term reward. Reinforcement, however, is the driving force that causes the associative connection between a particular response to (or effect of) a specific action that is not directly linked to the effect or response. Crucially reinforcement is not necessarily a pleasure related process in as much as unpleasant stimuli in conditioned avoidance paradigms will produce negative reinforcement just as readily as overtly pleasurable stimuli (or activation of the reward system) will produce (positive) reinforcement in, for example, the classical operant self-administration paradigm (for a review of reward and reinforcement see White, 1989).

A key neuronal component of the central reward pathway is the dopamine system, especially that of the medial forebrain bundle (Wise, 1998). This system projects from the substantia nigra and ventral tegmental area to the olfactory tubercle, medial prefrontal cortex, nucleus accumbens, and amygdala (Figure 1.2; Lindvall and Bjorkland, 1978; Iverson and Koob, 1977). Intracranial self-stimulation using an operant lever pressing model has demonstrated reinforced responding for electrical stimulation of diverse brain regions including the lateral hypothalamus (Olds, 1962),

nucleus accumbens (Rolls, 1971), olfactory tubercle (Prado-Alcala and Wise, 1984), septal area (Olds and Milner, 1954), hippocampus (Ursin *et al.*, 1966), amygdala (Wurtz and Olds, 1963) caudate-putamen (Phillips *et al.*, 1976) mid-thalamus (Cooper and Taylor, 1967) ventral hypothalamus (Ball, 1972), ventral tegmental area (Ward, 1960), and the prefrontal cortex (Routtenberg and Sloan, 1972). However, drugs of abuse, including ethanol, have been shown to increase lever-pressing for stimulation of brain regions limited more to the medial forebrain bundle regions, where the lateral hypothalamus, posterior hypothalamus and ventral tegmental area were most sensitive (Wise, 1998).

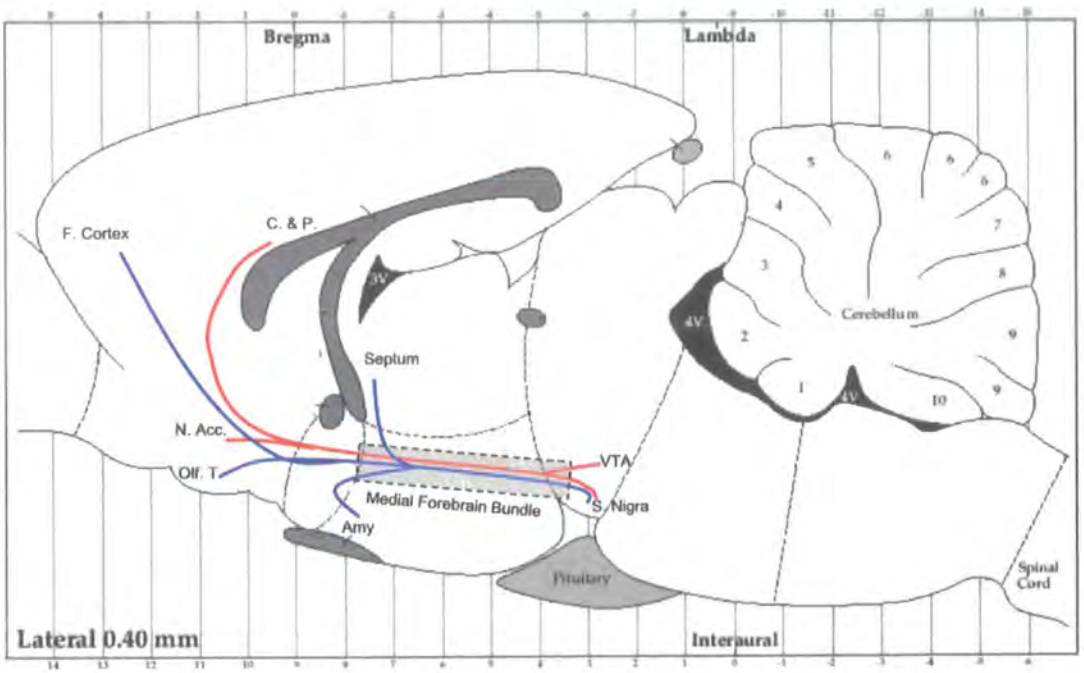
Further evidence for the role of the medial forebrain bundle dopamine neurons in reward is provided by 6 hydroxy-dopamine lesioning of the terminal regions of these projections (Quarfordt *et al.*, 1991). Lesioning of the dopaminergic cells projecting to the nucleus accumbens and olfactory tubercle significantly decreased ethanol preference in a two bottle (8% ethanol and water) preference experiment (Quarfordt *et al.*, 1991).

Figure 1.2: Diagrammatic representation of the major dopaminergic projection pathways involved in reward and reinforcement. Red pathway indicates the nigro-striatal projections and the blue pathway indicates the mesolimbic pathway.

S.Nigra = substantia nigra; VTA = ventral tegmental area; Amy = amygdala; N. Acc = nucleus accumbens; Olf. T. = olfactory tubercle; F. Cortex = frontal cortex; C & P = caudate and putamen of the striatum.

This diagram is an adaptation from Paxinos and Watson, 1986.

Figure 1.2: The primary dopaminergic projection pathways of the medial forebrain bundle.



However, while the medial forebrain bundle dopamine cells are the primary system for the rewarding properties of ethanol and drugs of abuse (Wise, 1998), the reinforcement of operant responding for ethanol was not altered by lesioning of the nucleus accumbens (Rassnick *et al.*, 1993).

In light of these findings, the VTA is considered to be a crucial area of interest for investigations into the molecular and cellular actions of ethanol that may lead to changes in ethanol consumption in the whole animal through modulation of the central reward system, as well as being a good candidate site for the activity of factors that may modulate ethanol's actions or control ethanol consumption.

Ethanol and the dopamine system

Acute ethanol

Ethanol exposure appears to decrease the dopamine receptor level with no change in affinity state in mesolimbic but not striatal membrane (Fuchs *et al.*, 1987) although the level and duration of ethanol exposure varies greatly between publications, and there is little consistency in the radio-labelled ligands used. However, one study investigating the relevance of the length of ethanol exposure demonstrated decreased receptor number after 1 and 4 weeks ethanol access, increased receptor number after 2 and 3 weeks ethanol access, by 6 and 10 weeks ethanol access there was no change in the dopamine receptor number (as measured by ^{3}H-SCH23390 binding; Hamdi and Prasad, 1993).

Acute systemic ethanol administration has been shown to cause increases in dopamine release in mesolimbic terminal regions (nucleus accumbens: Imperato and Di Chiarta, 1986; Samson *et al.*, 1997), while only doses of ethanol (2.5 and 5 g/kg i.p.) that were reported as sedative in this study, caused a significant increase in striatal dopamine release (Imperato and Di Chiara, 1986). Interestingly, direct application of ethanol to the nucleus accumbens had no effect on dopamine release

(Samson *et al.*, 1997), unless very high concentrations of ethanol (1.6mM – 1.6M) were present in the dialysis probe (Wozniak *et al.*, 1991).

Similar to the findings that systemic ethanol increased mesolimbic dopamine release, electrophysiological recordings of cellular activity in the medial forebrain bundle (recorded at the level of the VTA) demonstrated that acute ethanol dose - dependently increased firing of these neurons *in vitro* (Brodie *et al.*, 1990; Brodie *et al.*, 1995) and *in vivo* (Gessa *et al.*, 1985). Interestingly, significant activation of the VTA by ethanol required concentrations over 20mM *in vitro* (Brodie *et al.*, 1990) and the increase in cell firing by ethanol was not calcium sensitive, and was present in both brain slice and single cell preparations, indicating a direct effect of ethanol on the firing cell itself (Brodie *et al.*, 1990; Brodie *et al.*, 1995). Furthermore, Mereu *et al.* (1984) demonstrated that ethanol was twice as potent at activating the VTA than the substantia nigra dopamine cells *in vivo*.

These results indicate a specific action of ethanol on mesolimbic cell firing that further supports the idea that the mesolimbic system, and the VTA specifically, is an important target for ethanol's actions and that it may be part of a crucial mechanism behind ethanol reward and thus regulation of ethanol consumption

Chronic ethanol

Several studies have investigated the effect of chronic ethanol on dopamine release and metabolism, however the method of ethanol administration differs greatly between publications as does the time point of dopamine measurement (post-mortem; during ethanol exposure; various time points during ethanol withdrawal). However, the overall results seem to indicate that chronic ethanol exposure results in decreased post-mortem dopamine levels in the olfactory tubercle and medial pre-frontal cortex (Fadda *et al.* 1989, 1990), while microdialysis studies demonstrated increased release of dopamine in nucleus accumbens (Weiss *et al.*, 1993; Nurmi *et al.*, 1996) but not the

striatum (Diana *et al.*, 1992; Rossetti *et al.*, 1993). The difference between microdialysis results and post-mortem results can be readily explained by the difference in sampling method. The post-mortem measurements of dopamine used by Fadda *et al.* (1989, 1990) measured the dopamine remaining in brain homogenate (i.e. dopamine contained in vesicles and the synaptic terminal) whereas the microdialysis studies measured dopamine released from cells. If dopamine release was to increase significantly then the remaining cellular dopamine may diminish sufficiently to produce a decreased level of dopamine in the post-mortem sample (Fadda *et al.*, 1990).

These results further demonstrate a significant effect of ethanol on the mesolimbic aspect of the dopaminergic system, with far less role for ethanol in the nigro-striatal dopamine system. Furthermore, the dopamine release in the nucleus accumbens is thought to be primarily due to activity of the dopamine cells projecting from the VTA (Imperato and Di Chiara, 1986).

Withdrawal from chronic ethanol

Following withdrawal from chronic ethanol intake, dopamine release was decreased in the nucleus accumbens (Rossetti *et al.*, 1993; Weiss *et al.*, 1996) but not the striatum (Uzbay *et al.* , 1998; Eisenhofer *et al.*, 1990). However, the decreased dopamine release in the nucleus accumbens returned to baseline levels after 24 hours (Rossetti *et al.*, 1993) or reintroduction of ethanol (Weiss *et al.*, 1996). In line with the decreased accumbal dopamine release, VTA firing rate was significantly decreased after withdrawal from ethanol, although there was no decrease in the number of cells found (Diana *et al.*, 1992).

Interestingly, however, Diana *et al.* (1993) reported a sustained decrease in firing rate lasting up to 72 hours after withdrawal from ethanol. Bailey *et al.* (1998) demonstrated a depressed VTA firing rate 24 hours after withdrawal from chronic

ethanol that was still present (to a lesser degree) 6 days after withdrawal. Two months after withdrawal from ethanol, the VTA firing rate was not significantly different to ethanol naïve controls (Bailey *et al.* , 1998). Dopamine measured in a midbrain slice was also depressed 24 hours after ethanol withdrawal (Bailey *et al.*, 2000), but contrary to the results seen with VTA firing rate, dopamine release had returned to baseline by 6 days after ethanol withdrawal, and at 2 months after withdrawal, while dopamine release remained normal, dopamine metabolism had decreased (Bailey *et al.*, 2000).

These results offer a critical insight into important, long term effects of chronic ethanol exposure after withdrawal from ethanol consumption, and *in vivo* measurements of cellular activity and dopamine release provide a very clear picture of decreased VTA firing and decreased mesoaccumbal dopamine release with no prolonged effects in the nigrostriatal dopamine system.

The dopamine system clearly plays a very important role in ethanol reward and ethanol itself directly modulates the activity of the mesolimbic dopamine system resulting in adaptations to ethanol during chronic administration and altered cell firing and dopamine release following withdrawal from ethanol. The cells that release dopamine in the nucleus accumbens originate in the ventral tegmental area and so this area is clearly a very important target for the investigation of ethanol's mechanism of action and the interaction of other systems (such as stress hormones) with ethanol in modulating the firing rate of these cells.

The Excitatory Amino Acid System

The major excitatory input into the ventral tegmental area arise in the medial prefrontal cortex, amygdala and the brain stem, primarily releasing glutamate which acts via ionotropic receptors to increase cell firing rate and regulate burst firing of the mesolimbic dopamine cells (Johnson and North, 1992).

The ionotropic receptors are divided into three groups on the basis of their sensitivity to exogenous agonists (Davies *et al.*, 1979): N-methyl-D-aspartate (NMDA receptors), DL-α-3-hydroxy-5-methylisoxazole-4-propionate (AMPA receptors) and kainate receptors. Each of these three glutamate receptor subtypes is constructed from multiple subunits. NMDA receptors are assembled from various combinations of NR1 and NR2 subunits. AMPA receptors comprise Glu1-7 subunits and kainate receptors KA1-2 subunits (Nakanishi, 1992). These subunits are collected together to form numerous different heteromeric receptors which can be distinct in their ligand gating properties, modulation and function. These receptors are ubiquitously expressed throughout the CNS, and so a key factor for the regional effects of ethanol on the glutamate system appears to be the regional distribution of the subunits within each receptor complex.

The Effects of Acute Ethanol on the glutamate system

Acute Ethanol and the NMDA receptor complex

Acute ethanol has a great diversity of effects on neuronal cells and the NMDA receptor complex. *In vitro* application of acute intoxicating concentrations of ethanol (up to 50mM) inhibited stimulated calcium influx (Hoffman *et al*, 1989), NMDA induced increases in intracellular calcium (Dildy and Leslie, 1989), NMDA receptor activated ion currents in hippocampal neurons (Lovinger *et al.*, 1989), NMDA evoked catecholamine, acetlycholine and noradrenaline release from cortex and striatum

(Gothert and Fink, 1989) and NMDA induced excitotoxicity (Lustig *et al.*, 1992). Ethanol (25-100mM) also selectively attenuated hippocampal release of glutamate and aspartate on application of K^+ (Martin and Swartzwelder, 1992). However, *in vivo* application of acute ethanol has very region specific effects. NMDA-evoked neuronal activity in the inferior colliculus and hippocampus was potently inhibited by iontophoretic application of ethanol, but not NMDA-induced activity of the lateral septum (Simson *et al.*, 1993). Acute iontophoretic application of ethanol also inhibited NMDA-induced activity in the medial septum (Simson *et al.*, 1991) as well as NMDA- quisqualate- and glutamate-induced excitation of the locus coeruleus (Engberg and Hajos, 1992) and NMDA-induced seizure activity (Kulkarni *et al.*, 1990).

The site of action of ethanol is not clear, only the sites where ethanol does not exert its effects. Ethanol's effects do not appear to be mediated by direct activity inside the channel (Reynolds and Rush, 1990), nor does it compete at the NMDA binding site (Rabe and Tabakoff, 1990; Dildy-Mayfield and Leslie, 1991) on the ion channel (Hoffmann *et al.*, 1989), at the Mg^{2+} site (Rabe and Tabakoff, 1990) or at the polyamine site (Matsumoto *et al.*,199*a*). There is some evidence that ethanol may alter the kinetics of channel opening (Snell *et al.*, 1993) and these effects of ethanol can be reversed by glycine application in some, but not all brain regions (cerebellar granule cells, Hoffmann *et al.*,1989; sometimes cortex and hippocampus, Snell *et al.*, 1993). However, this is not thought to be a competitive action but rather that ethanol (and *n*-alcohols) may bind to a hydrophobic pocket (Peoples and Weight, 1995).

Acute ethanol on non-NMDA glutamate receptors:

Both AMPA and kainate receptors are significantly less sensitive to ethanol than the NMDA receptor complex. Most effects of ethanol have only been observed with ethanol concentrations over 100mM which would be anaesthetic in a whole

animal (Hoffmann *et al.*, 1989; Lovinger, *et al.*, 1989; Dildy-Mayfield and Harris, 1992). Ethanol (over 100mM) is generally inhibitory in its actions at these receptors, attenuating kainate-induced noradrenaline release (Fink and Gohert, 1990), inhibiting kainate induced currents but only at low kainate concentrations (Dildy-Mayfield and Harris, 1992). However, the concentration used to elicit these responses leave some doubt as to whether they are physiologically relevant effects of ethanol in the study of ethanol dependence.

Acute ethanol on metabotropic glutamate receptors:

The role of the metabotropic glutamate receptors in ethanol dependence has not been investigated very widely and ethanol has been used primarily as a pharmacological tool along with the inhalational anaesthetics. Minakami *et al.* (1993) have shown that ethanol (20-120mM) inhibited glutamate activated, calcium dependent chloride currents in oöcytes expressing mGluR5 but not mGluR1. Groul *et al.* (1997) demonstrated that 33 and 66mM ethanol inhibited K^+ induced calcium release induced by mGluR actrivation but that 10mM ethanol had no effect. The few studies that have investigated the effects of ethanol on metabotropic glutamate receptors indicate a subtype specific inhibition at higher ethanol concentration than those active at NMDA receptors but lower than the effective concentrations at AMPA and kainate receptors. The metabotropic glutamate receptors play an important role in the modulation of neurotransmission, but much more work is necessary to elucidate if there is any real role for these receptors in the effects of physiological concentrations of ethanol in dependence.

The effects of chronic ethanol on the glutamate system

Chronic ethanol and the NMDA receptor ion channel complex

Chronic exposure to ethanol liquid diet (3.9-4.7 g/kg/24h) lead to up-regulation of the NMDA receptor and its responses (Chen *et al.*, 1997). The number of glutamate binding sites in synaptosomal preparations was increased by 4.2-4.5 g/kg/24h (liquid diet; Michaelis *et al.*, 1978). Binding of glutamate also increased following chronic *in vitro* ethanol (100mM; Snell *et al.*, 1993) but not that of glycine, while MK-801 binding increased in the hippocampus (Grant *et al.*, 1990; Sanna *et al.*, 1993, Snell *et al.*, 1993) but changed variably in the cortex, striatum and thalamus (Gulya *et al.*, 1991). *In vitro* studies also showed increases in NMDA/glycine induced calcium influx after 'chronic' incubation with 100mM ethanol (Iorio *et al.*, 1992) and cerebellar granule cells were sensitized to the excitotoxic effects of NMDA (Chandler *et al.*, 1993; Iorio *et al.*, 1993).

The overall effect of chronic ethanol is clearly an up-regulation of the NMDA receptors and the glutamate system as a reaction to the initially inhibitory phase of ethanol's actions on the glutamate system. As described earlier this up-regulated NMDA system may then lead to an overshoot in glutamatergic activity following withdrawal from chronic ethanol and this may explain some of the hyperexcitability and decreased seizure threshold observed during the physical withdrawal syndrome. The application of glutamate antagonists, such as glutamate diethyl ether (Freed and Michaelis, 1978), attenuated withdrawal behaviours, supporting the glutamatergic overshoot theory.

Ethanol withdrawal also potentiates NMDA-induced damage to the hippocampus (Davidson *et al.*, 1993, 1995) probably due to the up-regulated receptors that are no longer inhibited by the presence of ethanol. Importantly, ethanol withdrawal seizure sensitivity is maximal at 24-48 hours and the elevated MK-801 binding witnessed during chronic ethanol as a liquid diet for 7 days (ethanol intake

level not provided) decreased back to control levels over a similar timecourse (Gulya, *et al.*, 1991) as did elevations in the NR2 subunit mRNA (Follesa and Ticku, 1995).

Chronic ethanol on non-NMDA glutamate receptors

Chronic ethanol inhalation, resulting in blood ethanol levels of 1.4mg/ml, lead to a supersensitivity to kainate-induced seizures (Freed and Michaelis, 1978) but 100mM ethanol *in vitro* did not affect the level of kainate receptor binding sites in either the hippocampus or the cortex (Snell *et al.*, 1993). There appears to be no difference in subunit expression after chronic ethanol liquid diet (blood ethanol 46-65mM; Trevisan *et al.*, 1994) However, AMPA receptor mRNA transcription may be affected by chronic ethanol (20% v/v ethanol for 38 weeks; Bruckner, *et al.*, 1997) and repeated withdrawal from two days of ethanol intoxication was accompanied by decreased AMPA binding sites with no changes in NMDA or kainate binding sites (Ulrichsen *et al.*, 1996).

Beyond these findings, little is known about the non-NMDA glutamate receptors and their role in ethanol's actions, however a picture of increased sensitivity following chronic ethanol is slowly building and may indicate that these receptor systems are actually an important, although possibly subtle, factor in ethanol dependence.

Chronic ethanol and metabotropic glutamate receptors

Even less is known about the effects of chronic ethanol on the metabotropic glutamate receptors than is known about the acute effects of ethanol. Simonyi, *et al.*, (1996) have shown that chronic ethanol (6 g/kg/24h for 4 weeks) decreased the mRNA for mGluR1, yet the little acute data available indicates that this receptor subtype does not seem to mediate ethanol's effects. A great deal of investigation

remains to be done to elucidate the role of these receptors in the effects of ethanol on the excitatory amino acid systems.

Neuronal Calcium channels

Calcium channel subtypes and nomenclature

Calcium plays a vital role in the regulation of neuronal excitability and, while ligand-gated cation channels (eg, the NMDA sensitive glutamate receptor) show a distinct degree of calcium permeability, calcium is rarely the primary ion of conductance in these channels. Influx of calcium into neurons is more usually via the voltage operated calcium channels that are opened at depolarising membrane potentials. Since increased intracellular calcium is responsible for many key activities in neuronal cells (such as activation of neurotransmitter release by vesicular exocytosis, activation of many intracellular messenger systems including several kinases, and activation of the calcium sensitive potassium channels which then repolarise / hyperpolarise the cell membrane and close the voltage operated calcium channels) so specific, co-ordinated manipulation of these channels may be a useful tool for the modulation of neuronal responses and adaptations to chronic ethanol in the treatment of ethanol dependence.

There are currently 7 subtypes of voltage-operated calcium channel known (Walter and Messing, 1999) – L-type (ϖ-conotoxin sensitive and insensitive forms), N-type, P-type Q-type, R-type, T-type. Each subtype has characteristic differences in voltage range of activation, rate of deactivation, and pharmacological differences and differential distribution in the central nervous system and peripheral systems (Walter and Messing, 1999). The voltage-operated calcium channels are multimeric complexes of at least 3 subunits: α_1, $\alpha_2\delta$, and β (McClesky, 1994), however, much of the subtype diversity (pharmacological and physiological) is due to differences in the

α_1 subunit which contains the Ca^{2+} pore and binding sites for the channel selective antagonists.

Voltage operated calcium channels and ethanol

The effects of acute ethanol on voltage operated calcium channels

There is much evidence that several aspects of ethanol dependence could be attributed to the L-type calcium channels. These channels are the only dihydropyridine–sensitive subgroup and are thus blocked by compounds such as nimodipine, isradipine, nifedipine, and verapamil. The subdivision of the L-type channel into ϖ-conotoxin sensitive and insensitive subgroups is a relatively recent development in voltage operated calcium channel nomenclature (Walter and Messing, 1999) and thus much of the research into ethanol and the L-type channels has simply designated all L-type channels as dihydropyridine-sensitive and not subdivided this 'family'.

Acute ethanol (10-100mM) induced decreases in *in vitro* vasopressin release, which correlated (in rats) with inhibition of the L-type voltage-operated calcium channels attenuating neurohypophysis release of vasopressin (Wang *et al.*, 1991). The open probability of L-type channels was greatly reduced, primarily by shortening of the open duration of the channel, by 10-50mM ethanol (Wang *et al.*, 1994). Ethanol induced inhibition was greater when calcium currents were evoked in PC12 cells from a depolarized holding potential (Mullikin-Kilpatrick and Treistman, 1995). However, various G_i inhibitors (pertussis toxin, antibody against α_{i1} α_{i2}, guanosine-5'-*O*-(2-thio)diphosphate) applied to undifferentiated, but not neuronal growth factor-differentiated, PC12 cells reduced ethanol inhibition of L-type channels (Mullikin-Kilpatrick *et al.*, 1995) suggesting that ethanol acts on the channel directly and also via G_i interactions. Overall, ethanol inhibits L-type calcium channel activity, however

the mechanism appears to differ depending on the cell line used, and these results do not necessarily prove that L-type channels play a therapeutically useful role in ethanol intoxication.

The effects of chronic ethanol on voltage operated calcium channels

Chronic ethanol exposure has been shown to increase the density and function of neuronal (dihydropyridine sensitive) L-type channels both in cell culture (PC12 cells showed increased dihydropyridine binding and K^+ evoked $^{45}Ca^{2+}$ uptake; Messing *et al.*, 1986; Skattebol and Rabin, 1987) and in membranes from ethanol dependent rats (dihydropyridine calcium channel antagonist binding sites increased; Dolin *et al.*,1987; Brennan *et al.*, 1990; Guppy *et al.*, 1995). Studies using hippocampal slices from mice following chronic ethanol administration demonstrated that L-type calcium channels played a sginificant role in ethanol withdrawal-induced neuronal excitability (Whittington *et al.*, 1993). This up-regulation of the L-type channels may be important in the development of ethanol dependence, and during the withdrawal phase following chronic ethanol these up-regulated channels appear to contribute to the intense neuronal hyperexcitability reported.

L-type calcium channel antagonists have been shown to reduce withdrawal signs and the mortality in ethanol-dependent rodents deprived of ethanol (Little *et al.*, 1986; Bone *et al.*, 1989; Pucilowski *et al.*, 1989; Littleton *et al.*, 1990; Colombo *et al.*, 1995). Since physical withdrawal, and avoidance thereof, is thought to be associated with drug 'craving' and drug seeking, it is highly possible that L-type calcium channel up-regulation also plays an important role in the maintenance of consumption. Indeed, L-type calcium channel antagonists have been shown to reduce ethanol consumption in many animal studies (Rezvani and Janowsky, 1990; Rezvani *et al.*, 1991; Fadda *et al.*, 1992; Pucilowski *et al.*, 1992; De Beun *et al.*, 1996a; Gardell *et al.*, 1997). Mice selectively bred to be ethanol withdrawal sensitive and in-sensitive

showed high and low levels of ethanol-induced L-type channel up-regulation respectively (Brennan *et al.*, 1990).

Increased levels of dihydropyridine binding sites following chronic ethanol and the protection against the withdrawal syndrome by acute dihydropyridine administration indicate a significant role for L-type calcium channels in ethanol dependence. However, the findings that offer the greatest therapeutic interest relate to the chronic administration of dihydropyridines during chronic ethanol intake. Tolerance to the ataxic effects of ethanol when examined on the rotorod is readily achieved under experimental conditions. However, administration of nitrendipine (50 mg/kg) at the same time as chronic ethanol consumption (but not a single administration of nitrendipine on the test day) prevented the development of this tolerance (Dolin and Little, 1989). Chronic treatment with nitrendipine given concurrently with ethanol, prevented the ethanol withdrawal syndrome in mice even though the nitrendipine treatment was stopped 24 or 48h before the withdrawal testing (Whittington *et al.*, 1991). Nitrendipine was effective when given for 2 weeks, but not after only two days' treatment, and blocked the ethanol-induced increase in dihydropyridine binding sites as well as preventing the ethanol withdrawal syndrome. However, chronic nitrendipine had no effect on the seizure threshold to bicuculline (in animals that were not given ethanol) (Whittington *et al.*, 1991).

Whittington *et al.* (1991) also demonstrated that concomitant administration of isradipine with chronic ethanol prevented the elevated dihydropyridine binding sites reported by Dolin *et al.* (1987), whilst chronic nitrendipine combined with ethanol prevented ethanol-induced decreases in long-term potentiation in isolated hippocampal slices (Ripley and Little, 1995).

One hypothesis for the action of dihydropyridine compounds in protecting against adaptations to chronic ethanol (tolerance and withdrawal etc.) is that the dihydropyridine compounds may be interacting with the cytochrome P450 isoform

that is responsible for the microsomal oxidation of ethanol (isoform 2E1; Lieber, 1997). However, while dihydropyridines in general do interact with cytochrome P450 metabolic pathways their activity is primarily inhibitory (Katoh *et al.*, 2000) and they do not significantly alter the activity of the cytochrome P450 (2E1) isoform (Katoh *et al.*, 2000) suggesting no effect of dihydropyridines on ethanol metabolism.

These results provide evidence for a functional role for dihydropyridine-sensitive calcium channels in ethanol dependence and further implicate these calcium channels as a therapeutic target for protection against the ethanol withdrawal syndrome and some of the effects of chronic ethanol consumption (such as increased calcium channel number; Whittington *et al.*, 1991; and the resultant withdrawal hyperexcitability; Whittington and Little, 1991; Ripley *et al.*, 1996). However, the peripheral (especially cardiac) effects of calcium channel blockade are still a major hurdle or the development of these channels as a therapeutic target for the treatment of ethanol dependence. A clinical study from Shulman *et al.* (1998) however, indicated that both nifedipine and verapamil are potentially useful treatments for withdrawal and craving with ethanol dependence (as well as dependence on opiates, amphetamine, benzodiazepines.

There is little or no information on the effects of acute and chronic ethanol on the other types of voltage-operated calcium channels. What little information there is pertains to the neurohypophysis or the PC12 cell line and show no great effect of ethanol or role for these channels in ethanol consumption and dependence (Walter and Messing, 1999).

Stress and ethanol dependence

Defintion of stress as it pertains to this thesis.

Currently, the induction of stress in laboratory animals is considered to involve the stimulation of the hypothalamo-pituitary-adrenal (HPA) axis resulting in the adrenal release of glucocorticoids (Fleshner *et al.*, 1995). However, whilst this is very often the case, increased HPA axis activity has not been proven with every laboratory 'stressor' used and is not necessarily reliable when the same stressor is applied to animals in different situations (Vogt *et al.*, 1981), and so is not the best defining characteristic of stress induced in the laboratory.

Behavioural responses to stress are even less useful as defining characteristics since they are entirely dependent on the type of stimulus used. An animal experiencing an inescapable electric shock (foot-shock) will not (usually) display a defeat posture, while animals experiencing defeat through subordination in a social colony will display classical defeat postures, yet both animals are experiencing 'stress'.

One of the aspects of the use of stressful stimuli in the laboratory is the very fact that we wish to 'use' them more commonly than we wish to 'investigate' responses to them. Generally, 'stress' is induced in some highly controlled, usually physically painful or noxious manner and then the effect of 'stress' (as a generalised term) on a particular physiological, often neurological, model of a disorder or disease state is examined. However, there has to be some question raised as to the relevance of stressors that are not part of the natural environment experienced by an individual. Very rarely will an animal in the wild be presented with an unavoidable foot-shock, or hot plate, nor will they be trapped under similar conditions as in a restraint stress test. Thus there is limited validity to the physiological and behavioural effects of these particular stimuli in terms of modelling the global stress response for the species or

strain tested. Likewise these models of the effects of stress on disease states or disorders would not seem very good models of the human condition and the interplay of stressful life events and experiences on these same disease states and disorders. It seems more pertinent to model the role of stress on disease states in animals by using stressful stimuli which are both naturally occurring in the animal in the wild and also bear some relevance to the type of stressful stimuli experienced in the human condition.

With respect to this thesis, and the study of the role of stress in the modulation of ethanol consumption and the contribution of life-stress to the relapse phase of ethanol dependence, I feel that the relevant stress systems are based around the application of 'psychosocial stress', or the stress induced from social interactions. This is a highly naturalistic stressor, present in everyday life for any gregarious species undergoing the establishment or maintenance of a 'pecking order' and is also clearly applicable to much of the hierarchical life-stresses experienced by humans through the work place and in complex family structures. Thus the terms 'stress' and 'psychosocial stress' will be used throughout this thesis with the understanding that I will be discussing the relevance of the stress produced from social interaction (at various levels) as a model of human life-stress events and their influence on ethanol dependence, rather than the stress induced through the physical discomfort or distress implemented by manipulations of the laboratory environment (eg food deprivation, restraint stress, disruption of light cycle, foot-shock).

The role of 'stress' in ethanol consumption

Life-stress and human alcohol consumption

Psychosocial stress in the form of stressful life events or chronic stress through work or family situations is thought to play a major role in both the initiation of

ethanol abuse and dependence and the relapse to drinking post-treatment (Brown *et al.*, 1995). Significant levels of psychosocial stress, unrelated to alcohol or drug abuse experienced post-treatment was prevalent in the 56% of male patients that had relapsed to ethanol consumption 1 year after treatment (Brown *et al.*, 1995). This study also indicated that subjects with better 'coping' skills (eg, avoidance of ethanol, problem-focused, support seeking) remained abstinent for longer (Brown *et al.*, 1995). This fits in well with the three stage model of stress where the coping skills are an integral part of the 'processing variable' stage of stress and, in theory, individuals with adequate coping skills could 'divert' the input stimuli such that, instead of producing stress, they were channelled into the coping behaviours. Thus individuals that were better able to cope with the stress inputs would be able to limit some of the behavioural responses to that stress (eg relapse as a response to psychosocial stress). Brown *et al.* (1995) also addressed this issue in another manner by determining the psychosocial vulnerability of individuals and comparing this with relapse rates. They found that continued abstainers from alcohol had a significantly lower score on the measure of the difficulty they experienced when trying to deal with stressful life situations (assessed using self-reporting style questionnaires) than patients that relapsed.

The role of stress in the modulation of ethanol intake in rodents

Various different types of stress have been shown to increase the ethanol intake of rodents. Mills *et al.* (1977) demonstrated that random unavoidable electric shock during 1h long, daily, sessions significantly increased the ethanol intake of rats during the first 24h period immediately following the shock. The increased intake occurred with both 5% and 10% v/v ethanol. However, they did not find any effect of electric shock on the overall weekly mean intake of ethanol, but rather a temporal shift in intake to a peak in consumption directly following the exposure to the shock

stress. In a similar experiment Caplan and Puglisi (1986) demonstrated that unavoidable footshock resulted in decreased ethanol intake while the animals were left in the shock chamber, but an increase in intake when the animals were removed from the shock chamber and returned to their home cage. These authors saw the same, environmental context specific, increase in ethanol intake on return to the home cage following conflict stress (social defeat) or food deprivation, but no change in intake when the animals were left in the test cages (Caplan and Puglisi, 1986). These results indicate a significant role for environmental cues in the regulation of ethanol intake, although, the fact that stress increased ethanol intake only after removal of the animal from the stressor environment and return to the familiar home cage would imply that the increased ethanol intake was not necessarily a coping mechanism, or self-medication of an anxiolytic (ethanol) during the stressful experience, but rather a response to the stressor once the experience has finished. Whether ethanol was self-administered as a 'post-traumatic anxiolytic' or for pharmacological effects other than anxiolysis is not clear from this data however.

Maudsley rats exposed to a daily 'tailpinch stress' (repeated tail pinches) significantly increased their ethanol intake after 21 days of exposure to ethanol and stressor (Adams, 1995). Interestingly, female Maudsley Non-Reactive rats stressed in this manner did not show a significant increase in ethanol intake, although Maudsley reactive rats did (Adams, 1995). Lynch *et al.*, (1999) have also demonstrated a significant effect of artificial stressors on ethanol intake. These authors used a schedule of 15 minute periods of restraint stress interspersed with 4 hour periods of access to ethanol as the only drinking fluid. Restraint stress had no effect on the amount of ethanol consumed when ethanol was the only solution available, however, it did significantly increase the level of ethanol consumed during periods of free choice ethanol and water in a two bottle preference model (Lynch *et al.*, 1999). The

effect of restraint stress on free choice ethanol intake remained for the 75 days of the test period (i.e. after the end of the stress schedule) (Lynch *et al.*, 1999).

All of these studies laid the ground work for the effect of 'stress' on ethanol intake, but they all used 'artificial stressors', primarily based around noxious stimuli. As discussed above, however, due to the diversity of definitions of stress and the lack of a unifying definition, the stress used throughout this thesis was of a more naturalistic manner, namely that due to social interaction and conflict.

Studies using animals housed in mixed sex social groups and experiencing the natural stress of subordination in an active social hierarchy indicate that the results from the 'artificial stressors' do seem to hold true with the naturalistic 'psychosocial stress' of social interaction (Blanchard *et al.*, 1992; Ellison, 1987). The study of Ellison (1987) demonstrated that subordinate animals from mixed sex groups housed in a large multiple chambered enclosure consumed more ethanol than their dominant counterparts. Similar results were reported by Blanchard *et al.* (1992) using mixed sex groups of rats in a visible burrow system. The effect of subordinate status within group housed rats on ethanol intake was also reported by Wolffgramm and Heyne (1990). These authors used rats housed in groups of four in standard laboratory cages with access to water, 5, 10 and 20% ethanol and they reported high ethanol intake of subordinate rats relative to dominant cage mates along the same lines as Blanchard *et al.* (1992) and Ellison (1987). All of these studies relating social status to ethanol intake implicate the subordinate rank as the 'high stress' social position. This assumption is based on the fact that the subordinate animal receives frequent defeats (or threats thereof) and limited access to food and sexual partners. However, one could argue that the maintenance of the 'alpha' or dominant status in a colony is equally stressful, especially in terms of fighting (both aggressive domination, and continual defence of social status). The 'stress' level of the subordinate status has been confirmed in several different studies (dependent upon the definition of

physiological responses accepted as being indicative of stress). Further discussion of the interpretations of social status and the associated stress levels and physiological changes can be found in chapter 3.

The hypothalamo-pituitary-adrenal axis and corticosterone

One of the key aspects of the stress imposed on rodents in the laboratory is thought to be the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the result of this activation is the release of the circulating glucocorticoid hormone corticosterone (Selye, 1976). Corticosterone is secreted by the adrenal cortex as a result of descending hormonal stimulation (adrenocorticotrophic hormone) from the pituitary gland (figure 1.1). Circulating plasma corticosterone levels show a distinct circadian rhythm which follows closely with the transition between light and dark phases and the associated changes in activity level (Doe *et al.*, 1954; Guillemin *et al.*, 1959; Aschoff, 1963; Scheving and Pauly, 1966). In addition to the diurnal fluctuations of plasma corticosterone levels, further environmental inputs can cause fluctuations in corticosterone release. Exposure to a multitude of artificial stressors as well as naturalistic social stressors increases corticosterone release in rodents, while positive reinforcers (eg food, sexual partners, and drugs of abuse) also stimulate similar increases in corticosterone levels (Piazza and Le Moal, 1997).

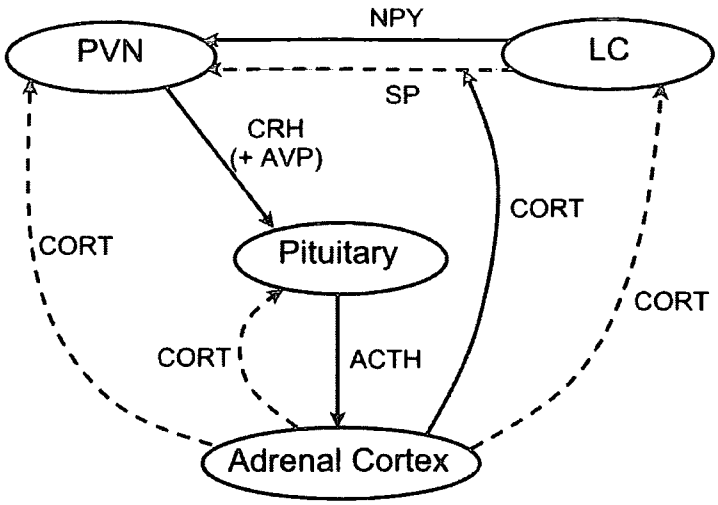


Figure 1.2: The hormonal communication of the hypothalamic-pituitary-adrenal axis.

The parvocellular corticotrophin releasing hormone (CRH) and arginine-vasopressin (AVP) neurons of the paraventricular nucleus (PVN) of the hypothalamus stimulate adrenocorticotrophic hormone (ACTH-corticotrophin) release from the Pituitary gland. ACTH stimulates the release of corticosterone (rodent) or cortisol (human) (CORT) from the adrenal cortex. CORT feeds back to the pituitary, PVN and also to the Locus Coeruleus (LC) regulation of the hypothalamus via Substance P (SP) and Neuropeptide Y (NPY). - - - → Inhibition ——→ Activation

There are two types of intracellular receptor mediating corticosterone's effects, primarily by the activation of specific transcription factors: mineralocorticoid (type I) and glucocorticoid (type II) receptors. Glucocorticoid receptors are a low affinity receptor (Kd for ^{3}H-corticosterone binding = 20.1nM; Sutanto and De Kloet, 1987) and are 88.7% and 97.6% occupied by high levels of circulating corticosterone during diurnal peaks or after stressful events respectively (Reul and de Kloet, 1985). Mineralocorticoid receptors have a high corticosterone affinity (Kd for ^{3}H-corticosterone binding = 0.9nM; Sutanto and De Kloet, 1987) and are 89.5% occupied even at the lowest basal levels of diurnal release (Reul and de Kloet, 1985).

An additional factor that is important in determinations of corticosterone levels in plasma samples is caused by the presence of the plasma proteins: corticosterone binding globulin and albumin. Both these plasma proteins bind corticosterone and play a major role in the differential bioavailability of corticosterone to the liver compared to other organs. Pardridge *et al.* (1983) demonstrated that ~75% of circulating corticosterone is bound to corticosterone binding globulin, ~20% is bound to albumin, and only ~5% is present as 'free'

corticosterone. Of particular importance in this dynamic equilibrium between the two binding proteins and the 'free' corticosterone is the penetration of corticosterone into the major organs. The capillaries of the brain contain tight endothelial junctions which prevent the plasma proteins from crossing the capillary wall (commonly described as the blood brain barrier). However, Pardridge *et al.* (1983) showed that the brain bioavailable corticosterone fraction was equivalent to the 'free' and albumin bound corticosterone (totalling ~26% of the circulating corticosterone), and that the fraction bound to corticosterone binding globulin did not penetrate into the brain. The same is thought to be true for all the major peripheral organs (except the liver) since, although the capillary walls do not contain the same level of tight endothelial junctions, the first pass penetration of plasma protein is still very low (Pardridge *et al.*, 1983). Interestingly, however, the liver bioavailable fraction was 2- to 4-fold greater than the brain bioavailable fraction in the study of Pardridge *et al.* (1983). This means that a large portion of the corticosterone bound to corticosterone binding globulin must cross the capillary walls into the liver in addition to the 'free' and albumin bound fraction, unlike in the other peripheral organs (Pardridge *et al.*, 1983).

The role of adrenocortical hormones in the modulation of ethanol intake

Removal of the adrenal glands, preventing any corticosterone release or synthesis, from Wistar rats resulted in a significant decrease in their ethanol preference (Fahlke *et al.*, 1994a). However, the replacement of corticosterone in these animals returned the ethanol preference to pre-adrenalectomy levels. Sham operated rats had no change in ethanol intake following surgery or corticosterone injection (Fahlke, *et al.*, 1994a). The mineralocorticoid, aldosterone, had no effect on the ethanol intake of adrenalectomised rats. These results imply a significant role for the glucocorticoid (but not necessarily the mineralocorticoid system) in the

maintenance of ethanol consumption. The authors measured the plasma corticosterone levels after adrenalectomy and showed a 90 fold decrease in the levels of circulating corticosterone. However, the remaining 0.2ug/100ml (~6nM) free corticosterone in the plasma may have been sufficient to bind to the high affinity mineralocorticoid receptors, thus limiting the activation of these receptors by aldosterone and so masking any potential effects on ethanol intake. In light of this, the results of Fahlke *et al.*(1994a) do not necessarily prove that mineralocorticoid receptors play no role in regulation of ethanol consumption, however, their results do clearly demonstrate a significant role for corticosterone in the maintenance of ethanol consumption.

Further investigations by the same group demonstrated that blockade of corticosterone synthesis with metyrapone decreased the ethanol intake of high preferring (> 60% of fluid drunk as ethanol) Wistar rats but not that of low preferring (< 30% of fluid drunk as ethanol) rats (Fahlke *et al.*, 1994b). Termination of metyrapone injections after 4 days produced an immediate return to the pre-treatment level of ethanol intake. Co-administration of corticosterone with metyrapone partially cancelled the effect of metyrapone on the ethanol intake of high preferring rats (Fahlke *et al.*, 1994b). These results also support the idea that corticosterone plays a vital role in the maintenance of ethanol consumption.

Interestingly the ethanol intake of low preferring animals was not affected by adrenalectomy in either study and sham operated animals showed no effect of corticosterone on ethanol intake. The presence of corticosterone may play a role in the maintenance of ethanol intake but is perhaps not the main regulatory drive for elevated ethanol intake, since increasing corticosterone levels in sham operated animals did not elevate their ethanol intake (Fahlke *et al.*, 1994a).

The longer term effects of surgical adrenalectomy have been investigated in Wistar rats (Fahlke *et al.*, 1995). Following access to ethanol in a two bottle preference model (6% v/v ethanol and water) for 1 week, rats were adrenalectomised and continuing ethanol intake was measured for 1 week. During this period the ethanol intake of adrenalectomised, but not sham operated, animals decreased significantly. However, during the subsequent three weeks the level of ethanol intake returned to pre-operative levels. After 1 week of ethanol access following removal of the adrenal glands, animals were chronically implanted with a corticosterone pellet restoring corticosterone plasma levels to the 24h mean concentration for the adrenally intact animals. The presence of steady levels of corticosterone for three weeks restored the ethanol intake of adrenalectomised animals, but also enhanced the levels of ethanol intake to above those of the intact animals (Fahlke *et al.*, 1995).

These results, while confirming the role of corticosterone in the maintenance of ethanol consumption also bring up two other issues. Firstly, the return to pre-operative ethanol intake levels after several weeks without adrenal glands indicates that, whilst corticosterone plays a significant role in the maintenance of ethanol intake, it is not essential. I would suggest that this recovery of ethanol intake in the absence of adrenal glands, and thus corticosterone, indicates that corticosterone is probably not having a direct effect on ethanol intake but rather is modulating other systems (i.e. affecting ethanol consumption indirectly). If this were the case then the slow recovery of ethanol consumption in the absence of corticosterone could be explained by the gradual adjustment of the direct control mechanism to the lack of corticosterone.

The second point raised by the study of Fahlke *et al.* (1995) was that replacement of corticosterone in a long term manner, but at a constant level, resulted in the enhancement of ethanol intake above that of intact control animals. These

results suggest, unlike the results from the acute study with replacement of corticosterone by repeated injections (Fahlke *et al.*, 1994 a,b), that constantly elevated corticosterone can exert a stimulatory effect on ethanol consumption but that the cyclical nature of circulating corticosterone levels does not, perhaps, allow for this effect. These results would suggest then that a stressor that induced an elevation in basal corticosterone release over a protracted period of time such as that produced by restraint stress (Fleshner *et al.*, 1995) or the stress of subordination (see chapter 3) could contribute towards an elevated ethanol intake. However, the lack of effect of adrenalectomy or corticosterone on the ethanol intake of low preference animals indicates that the HPA axis does not provide the ultimate control over ethanol intake and is not necessarily sufficient to initiate drinking in individuals that are not pre-disposed to (or have experience with) high ethanol consumption. Rather it seems that the HPA axis provides an important contribution to the complex network of systems modulating ethanol consumption.

General Hypothesis and Aims

In light of the reported effects of artificial stressors on ethanol intake and reinstatement of self-administration and the likelihood that stress is a significant factor in human relapse to alcoholism the studies presented here were designed to investigate the hypothesis that:

stress, specifically social stress, and the rodent hormonal response, corticosterone, plays a significant role in the modulation of ethanol intake and the cellular actions of ethanol on the dopamine reward pathway.

Several different studies were designed that used three different animal strains (two mouse, one rat). Each strain was chosen based on the previous data reported with these strains in the area of investigation and the suitability of these strains for the methods used.

The aim of these studies was to investigate whether:

1) social status was a significant factor in the level of ethanol consumption of individual animals housed in groups of four with access to three ethanol concentrations and water for over 12 months.

2) a single or repeated aggressive social defeat in a resident intruder paradigm would have any effect on the ethanol consumption of low preference, 'stresss-sensitive' C57.

3) corticosterone applied directly to the dopaminergic neurons of the midbrain reward system would effect either NMDA or ethanol induced increases in basal firing rate.

In addition to these studies designed to investigate the interplay of stress and the hormones of the HPA axis with ethanol's actions, the four bottle choice model of ethanol access described by Wolffgramm and Heyne (1995) was also validated using two test groups and the dihydropyridine calcium channel inhibitor, nimodipine. As discussed earlier, the calcium channel inhibitors have been shown to have a significant effect on ethanol preference, consumption, withdrawal and tolerance (page31), and the hypothesis was that, if the four bottle preference model was of use in the modelling of ethanol dependence then nimodipine would produce similar decreases in ethanol preference and consumption in this model as previously reported with two bottle preference models (Rezvani and Janowsky, 1990; Rezvani *et al.*, 1991; Fadda *et al.*,1992; De Beun *et al.*, 1996a).

Chapter 2

Free choice ethanol access and the effect of the dihydropyridine calcium channel blocker nimodipine.

Introduction:

Models of ethanol consumption: the four bottle preference.

In order to investigate the role of environmental and physiological systems in alcohol addiction, suitable experimental models of the human condition are necessary. As discussed in the overall introduction to this thesis, diagnosis of substance dependence requires the meeting of at least three of seven criteria (DSM-IV criteria page 214). A good animal model of alcohol dependence should also meet several of these diagnostic criteria. Both tolerance to the behavioural effects of ethanol and a physical withdrawal syndrome are readily elicited in simple animal models of free choice ethanol drinking and forced ethanol consumption. However, these criteria are not necessarily the best indicators of dependence since many drugs produce either tolerance to their effects, or a physical withdrawal syndrome on removal of the drug yet meet none of the other criteria for substance dependence.

However, withdrawal and tolerance are no longer considered to be the key characteristics of substance dependence (as discussed earlier; page 6) and so newer models of alcohol administration are needed that bring together the more pertinent behavioural characteristics of dependence of:

a) Long term intake showing the similar phases of controlled and uncontrolled intake of human alcohol abuse and dependence respectively.

b) Elevated ethanol intake following removal of alcohol and reintroduction (relapse), particularly after multiple abstinence and relapse phases.

c) Aggressive pursuit of alcohol despite aversive effects, both physiological and social (behaviour detrimental to family, work etc.).

Some of these aspects are reproduced by various different models of alcohol consumption in current use. Rats trained to respond for ethanol in a skinner box will, following chronic pre-treatment with ethanol, press a lever far more times for a single reward than a non-dependent animal. Following chronic alcohol intake, rats will clearly demonstrate an alcohol deprivation effect (ADE), even many months after the withdrawal syndrome has passed (Sinclair and Senter, 1967). Interestingly, up until Wolffgramm and Heyne (1995), no models had been reported that combined all the necessary factors of alcohol dependence into one model. Wolffgramm and Heyne (1995) reviewed a model of alcohol access in which Wistar rats were housed in groups of 4 and given 12-20 months continuous free access to tap water 5, 10, and 20 % (v/v) ethanol. From this model they reported a high level of ethanol intake (2.2 g/kg/24h estimated from Figure 8 in their publication) which was stable over the first 6 months of access (Wolffgramm and Heyne, 1995). From 6 months onwards, the ethanol consumption gradually increased in what the authors interpreted as an 'uncontrolled phase' of drinking. This increased intake was accompanied by a change in preferred alcohol solution from the 5% solution to the 10% solution as ethanol intake increased. Wolffgramm and Heyne correlated this phase with the loss of control seen in human alcoholics as they make the transition from alcohol abuse to dependence. Once this uncontrolled intake was established, they observed a mild withdrawal syndrome on removal of the alcohol solutions (possibly measured by hypersensitivity to foot-shock although the authors do not state that this was the method used, nor do they show any data). After 6 – 9 months abstinence the authors

reported a clearly elevated intake on reintroduction to the three ethanol concentrations and water (Wolffgramm and Heyne, 1995). One of the key findings of this study, beyond the characterisation of the physical dependence of the animals, was that the high ethanol preference exhibited in this model was not affected by the adulteration of the ethanol solutions with the aversive flavour of quinine. This strongly suggests that the animals are drinking ethanol for its pharmacological effects and not as a flavoured solution (Wolffgramm and Heyne, 1995).

The four bottle free choice model of Wolffgramm and Heyne (1995) reportedly meets the criteria of uncontrolled drinking; alcohol deprivation effect; a physical withdrawal syndrome; and a drive for alcohol despite aversive stimuli. However, there are no reports from any other laboratories on this method of ethanol access as presented by Wolffgramm and Heyne (1995), although the four bottle preference has been used in conjunction with repeated abstinence periods of three days once a month (Spanagel *et al.*, 1996).

The effect of voltage sensitive calcium channels in the modulation of ethanol intake in the four bottle preference model.

Dihydropyridine calcium channel antagonists have been shown to affect many different aspects of alcoholism, both at a cellular and behavioural level. Ethanol administered acutely to rat brain synaptosomes (Rius *et al.*, 1987) or chronically by inhalation to Sprague-Dawley rats (Dolin *et al.*, 1987) increased the maximal dihydropyridine binding (B_{max}). *In vitro* ethanol administration decreased depolarisation -induced $^{45}Ca^{2+}$ entry into synaptosomes (Harris. and Hood, 1980), and subsequent neurotransmitter release (Lynch and Littleton, 1983).

Alcohol preference has been extensively used as a measure of ethanol self-administration. Our laboratory has previously shown an effect of nimodipine on the ethanol preference of C57 mice. In a two bottle preference model, mice with access to 8% ethanol and water showed a decreased ethanol preference on the first day of administration of 2 and 10 mg/kg nimodipine, whilst 50 mg/kg reduced ethanol preference over 4 days (Smith *et al.*, 1999). Operant responding of Lister rats for ethanol was decreased by 10 mg/kg nimodipine, whilst 50 mg/kg nimodipine had little effect on responding for ethanol but decreased sucrose self-administration (Smith *et al.*, 1999). De Beun *et al.* (1996a) showed that 7.5 to 30 mg/kg nimodipine decreased the 8% ethanol preference of ethanol preferring rats. Ethanol intake and food consumption were also decreased but not total fluid intake. The dihydropyridine sensitive calcium channel agonist, BAYK8644, also decreased ethanol preference. However, this effect was not reversed by antagonist (nimodipine) administration and thus may not be due to a specific calcium channel effect of the agonist (De Beun *et al.*, 1996b).

Concomitant administration of nitrendipine with chronic ethanol intake prevented the physical withdrawal syndrome (Whittington *et al.*, 1991), protected against the increase in dihydropyridine binding sites induced by chronic ethanol (Whittington *et al.*, 1991) and prevented ethanol-induced decreases in hippocampal long-term potentiation (Ripley and Little, 1995).

Despite this clear evidence of a role for calcium channels in various aspects of ethanol consumption and withdrawal, the effects of calcium channel blockade by dihydropyridines has not been investigated in models of long term ethanol access nor in a model of ethanol preference using 3 ethanol concentrations and water. This study

was designed to demonstrate whether there was any effect of nimodipine administration on ethanol preference in a four bottle choice, and also to investigate any effects of dihydropyridine compounds on the elevated ethanol intake associated with reintroduction to ethanol following withdrawal from chronic ethanol consumption and a period of abstinence (i.e. the alcohol deprivation effect discussed in the earlier part of this chapter).

The effect of the calcium channel antagonist, nimodipine, on first time, continuous and 'relapse' ethanol drinking after long term ethanol consumption.

Aim:

The aim of this study was to investigate whether nimodipine had any effect on the ethanol intake of group housed male Wistar rats with 62 weeks experience of ethanol access as 5,10 and 20% v/v ethanol and tap water, or on the ethanol intake of these animals after 2 weeks abstinence from ethanol access in this model.

Methods :

Four bottle choice of ethanol consumption

Two groups of male Wistar rats were housed in groups of four in cages 60 x 38 x 20 cm, on a normal phase light cycle (12h/12h light/dark, lights on at 7am). Room temperature was maintained at 20°C, and the humidity between 40 and 60%. Standard laboratory chow was available *ad libitum* unless otherwise stated. The first group (Group A) consisted of 72 animals aged six to seven weeks at the beginning of the experiment, with starting weights of 194 ± 1.3g. These were given a continuous free choice between tap water and ethanol 5, 10 and 20% v/v for 82 weeks in order to investigate the pattern of ethanol drinking in this chronic ethanol model in our laboratory. The second group (Group B) contained 72 animals, 7 weeks of age at the start of the study (starting weights of 253 ± 1.9 g) that were given access to the same four fluids for 62 weeks, prior to inclusion in the nimodipine study following this section. Three control animals from this group died during the long term drinking period. The second group (Group B) was started 4 months after Group A in order to

control for any seasonal effects on ethanol acceptance and intake.

Both groups were split to control and drinking animals in the same manner, 12 of the 18 cages of rats (48 animals), were allowed continuous access to the choice of water, 5, 10 and 20 % v/v ethanol, while the remaining six cages (24 animals) had four bottles of water (control). This uneven split between controls and ethanol experienced animals was designed to provide a larger number of ethanol experienced animals for later studies.

All animals had continuous access to standard laboratory chow. Every three weeks the mean 24h ethanol and total fluid consumption was determined by measurements on three consecutive days, providing two sets of 24h consumption values. The results were expressed as g of ethanol/kg animal weight/24h (g/kg/24h) and the mean of the two 24h measurement periods for each cage was used to calculate the mean value across cages. In addition, for each ethanol concentration the percentage preference was calculated, as the amount of ethanol consumed as a percentage of the total fluid consumed. (One set of measurements, for week 62, was lost for both groups owing to computer failure).

Measurement of the physical withdrawal syndrome (Group A)

To examine the effects of ethanol withdrawal, the bottles of ethanol were removed from 12 rats after 55 weeks of drinking and the withdrawal behaviour was rated on a scale of 0 – 5 according to the criteria in Table 2.1. Behaviour was observed in the following three groups: (i) 12 rats from which the ethanol was removed (ii) 12 rats which continued to have access to the three ethanol concentrations and water (iii) 12 control animals with continuous access to water only. Ratings of behaviour were made every 30 min for 12 h, following the removal

of ethanol at 8 a.m., by an observer who was unaware of the prior treatment. The animals remained in their home cages; food was removed from all animals for the 12h and water was continuously available throughout.

Table 2.1 Behavioural ratings used for analysis of physical withdrawal syndrome.

Behavioural Ratings	
0 =	normal
1 =	increased reactivity to presence of observer.
2 =	myoclonic jerks
3 =	head twitches
4 =	head and body twitches
5 =	clonic seizure

Effects of 2 weeks ethanol deprivation and reintroduction (Group A)

The consequences of ethanol deprivation were studied on group A, in the 12 animals that were used in the withdrawal experiment. These were given access to just water and food for 2 weeks after withdrawal of the ethanol. After this period of forced abstinence, the animals were reintroduced to ethanol with the same 4 bottle choice. Ethanol consumption was measured once daily for the first 10 days of the reintroduction period.

Statistical Analysis:

When results were compared over several days for two or more treatments, two way analysis of variance was used with one within-subjects factor (time) and one between subjects factor (treatment), with Student-Newman-Keuls as a post hoc test. Where comparison was made over more than one day, within a treatment group, one way analysis of variance was used with Student-Newman-Keuls post hoc test. For

comparisons between two treatment groups on a single day, or between two days for the same group, Student's t-test was used.

The effect of nimodipine on ethanol preference:

The male Wistar rats of Group B were used for this experiment. After 62 weeks of free alcohol consumption the original groups were separated and housed individually in the same size cages as for the group housing period (60 x 38 x 20cm). Animals were housed at 20°C, humidity regulated to 40 – 60% and on a normal phase light cycle (lights on 0700; off 1900 h). Standard laboratory chow was available *ad libitum*.

Once singly housed the animals received the same fluids as before separation, (controls received water, ethanol experienced received 5, 10 and 20% (v/v) ethanol and water) experimental days were numbered from this first day of individual housing (day 1) forwards. Intake measurements were taken for 3 days (days 1 – 3) to provide a baseline intake value for individual housing. After the three baseline days the animals were split into the three groups listed below (also see Table 2.2). Ethanol and water intake were recorded daily for the entire experimental period.

i) water group – control animals (n=20) that previously drank just water, were given access to water only for the first 14 days after baseline (days 4 to 17), then the four bottle choice of water, 5, 10 and 20% (v/v) ethanol for 6 days (days 18 – 23).

ii) ethanol withdrawal group- half the animals previously drinking ethanol (n=21) were withdrawn and given four bottles of water for 14 days after baseline (days 4 – 17) then returned to the four bottle choice of water, 5, 10 and 20% (v/v) ethanol for the next 6 days (days 18 – 23).

iii) continuous ethanol group - the remainder of the animals previously drinking ethanol (n = 19) were given continuous access to the four bottle choice of water, 5, 10, and 20% (v/v) ethanol for the next 20 days after baseline (days 4 – 23).

Daily intraperitoneal injections of nimodipine or its vehicle (Tween 80, 0.5% in distilled water) were given from Day 10 to Day 23. The rats in each of the three drinking conditions (water, ethanol withdrawal and continuous ethanol) were divided into 3 groups, which received either 5 mg/kg nimodipine, 20 mg/kg nimodipine, or Tween vehicle. Injections were given 1h before the start of the dark phase so that the 8-12h duration of action of nimodipine would overlap with the peak drinking period after the animals awoke. Nimodipine doses were chosen based on the work of Smith *et al.* (1999) using Lister rats and De Beun *et al.* (1996b) using ethanol preferring rats. These studies implicated 20mg/kg as a dose likely to give a high to maximal effect, while 5mg/kg was implicated as a dose producing far less effect (if any at all).

Statisitical Analysis:

When results were compared over several days for two or more treatments, two way analysis of variance was used with one within-subjects factor (time) and one between subjects factor (treatment), with Student-Newman-Keuls as a post hoc test. Where comparison was made over more than one day, within a treatment group, one way analysis of variance was carried out, followed by Student-Newman-Keuls post hoc test. For comparisons between two treatment groups on a single day, or between two days for the same group, Student's t-test was used.

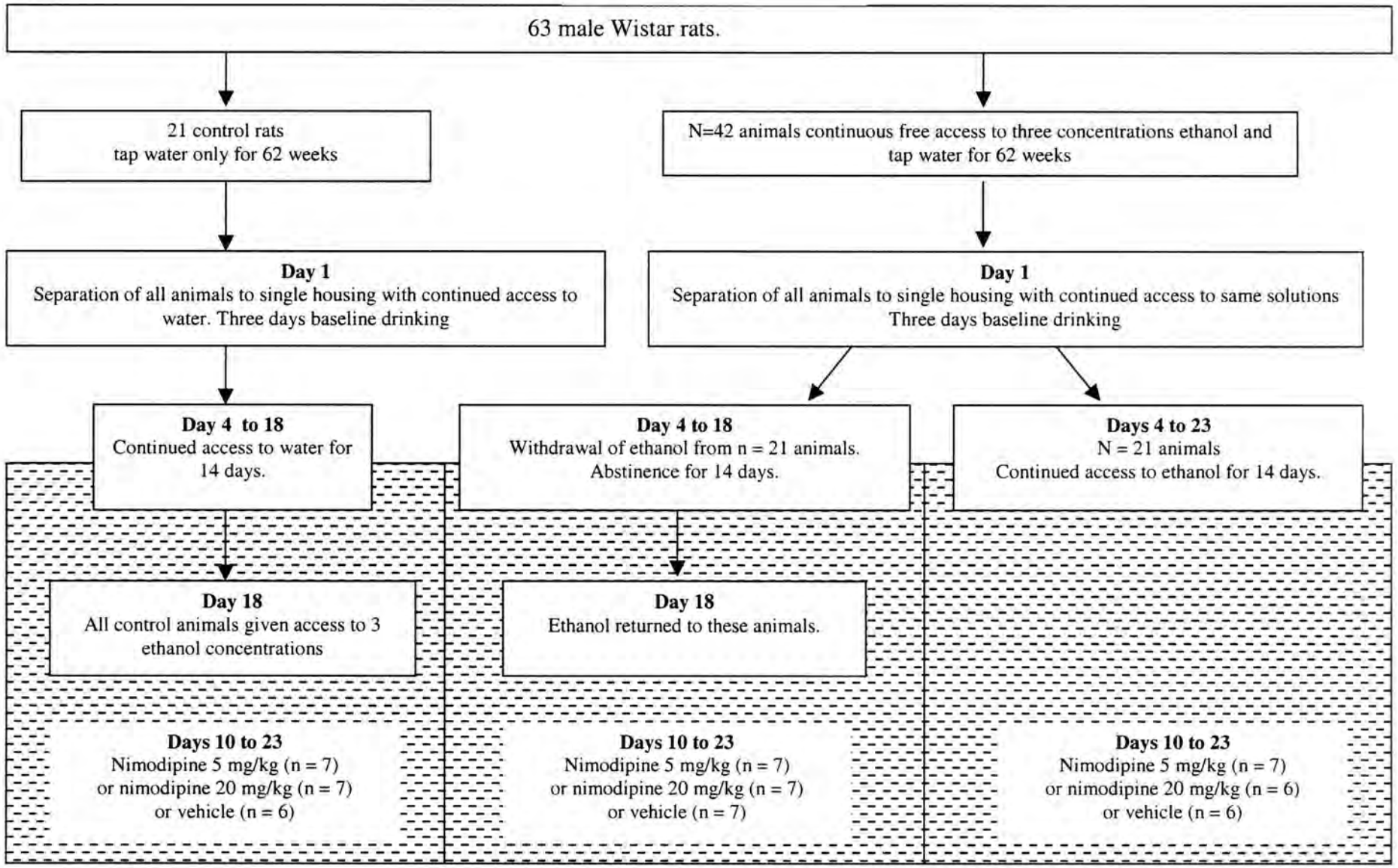
Prior to the start of the experiments it was decided that pairwise comparisons would be made only within the following treatment groups between the ethanol consumption

on Day 9 and on Day 10 (i.e. before and after the first dose of nimodipine) for the continuously drinking animals receiving 5 mg/kg nimodipine, those receiving 20 mg/kg nimodipine and those receiving Tween vehicle .

The F-test was used to compare the variances in the measurements of ethanol consumption. These comparisons were made for the ethanol consumption in each treatment group, at different times, as indicated in the Results section. Where the F test gave significant differences in the variance, pairwise comparisons were made by the Mann-Whitney U test.

Table 2.2: Flow diagram representing the experimental groups used. One large 'parent group' of animals (n=63) was given long term access to ethanol (5, 10 and 20% v/v) and/or water, prior to subdivision into withdrawal and reintroduction subgroups. These subgroups were also subdivided into nimodipine (5 and 20mg/kg) and Tween vehicle treatment groups.

Table 2.2


63 male Wistar rats.
21 control rats
tap water only for 62 weeks
N=42 animals continuous free access to three concentrations ethanol and tap water for 62 weeks
Day 1
Separation of all animals to single housing with continued access to water. Three days baseline drinking
Day 1
Separation of all animals to single housing with continued access to same solutions Three days baseline drinking
Day 4 to 18
Continued access to water for 14 days.
Day 4 to 18
Withdrawal of ethanol from n = 21 animals.
Abstinence for 14 days.
Days 4 to 23
N = 21 animals
Continued access to ethanol for 14 days.
Day 18
All control animals given access to 3 ethanol concentrations
Day 18
Ethanol returned to these animals.
Days 10 to 23
Nimodipine 5 mg/kg (n = 7)
or nimodipine 20 mg/kg (n = 7)
or vehicle (n = 6)
Days 10 to 23
Nimodipine 5 mg/kg (n = 7)
or nimodipine 20 mg/kg (n = 7)
or vehicle (n = 7)
Days 10 to 23
Nimodipine 5 mg/kg (n = 7)
or nimodipine 20 mg/kg (n = 6)
or vehicle (n = 6)

Results:

Four bottle choice model of ethanol consumption

The consumption of both group A and B has been presented as one data set. The mean ethanol intake across all cages was 1.56 ± 0.05 g/kg/24h, and this intake did not change significantly through the course of the experimental period (82 weeks; Figure 2.1). Ethanol preference did not change throughout the experiment either (Figure 2.2). There was an approximately 6 fold higher preference for the 5% solution than the 10% or the 20% ethanol solution ($P < 0.0001$) and there was no change in any of these preferences.

Withdrawal behaviour (Group A)

No signs of physical withdrawal were seen during the 12h observation period in the animals that were withdrawn from ethanol after 55 weeks of continuous access (data not shown as all behavioural ratings were 0 - normal).

Reintroduction to ethanol (Group A)

On the first day of reintroduction to ethanol the previously abstinent animals consumed significantly more ethanol than the continuous access controls on the same day (3.4 ± 0.12 g/kg/24h reintroduced group; 1.6 ± 0.37 g/kg/24h control; $P<0.005$; Figure 2.3). For the first six days of the reintroduction period, the consumption by the animals reintroduced to ethanol was higher than that of the animals that had continuous ethanol access ($P< 0.05$ for comparison on each of the six days after reintroduction). On the first day of reintroduction (Day 15), animals that had been abstinent for 14 days showed a significantly higher ethanol intake than on any other day subsequent to reintroduction (Days 16 - 24) ($P < 0.005$). No corresponding difference was seen in the animals that had uninterrupted access to the ethanol choice.

The animals from Group A with continuous access to ethanol were used for the study of the relevance of social status to group housed ethanol access (Chapter 3).

Figure 2.1: Wistar rats were housed in groups of four and given continuous free access to ethanol (5, 10, and 20% v/v) and water. Ethanol intake was measured every three weeks for 82 weeks. Data is presented as g of ethanol consumed / kg animal weight / 24 h. n = 96.

Figure 2.2: Wistar rats were housed in groups of four and given continuous free access to ethanol (5, 10, and 20% v/v) and water. Ethanol intake was measured every three weeks for 82 weeks. Data is presented as the 'percent preference' for each ethanol concentration: g of ethanol consumed / g total fluid consumed x 100. n = 96.

Figure 2.1: Ethanol intake of group housed Wistar rats with access to 5%, 10% and 20% ethanol (v/v) and tap water.

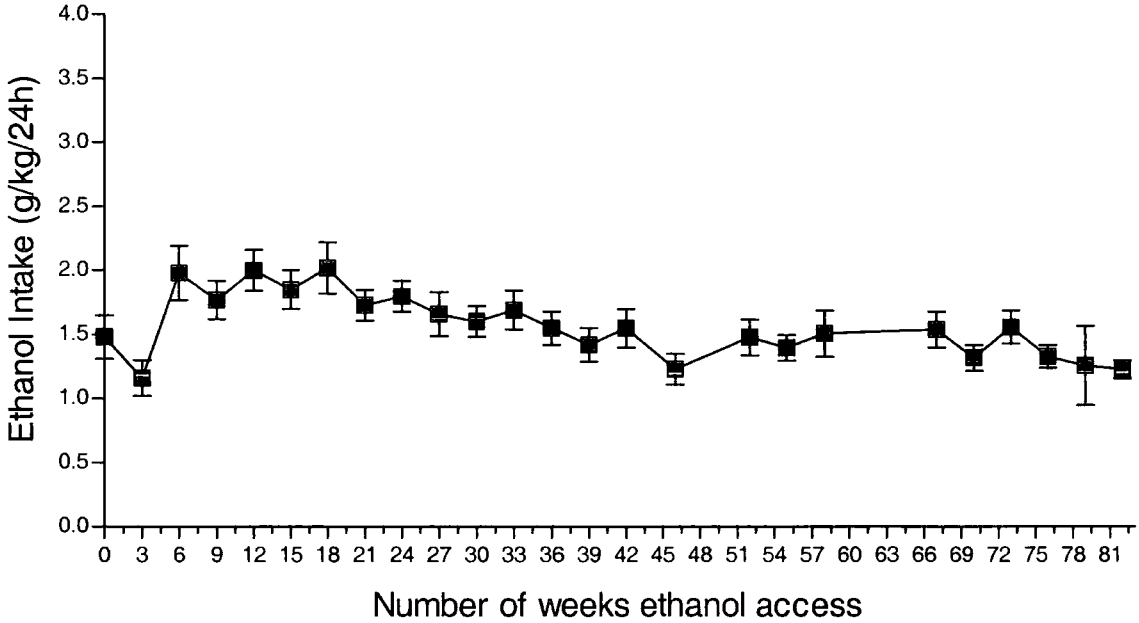


Figure 2.2: Percentage ethanol preference of group housed Wistar rats with free access to 5%, 10% & 20% ethanol (v/v) and tap water.

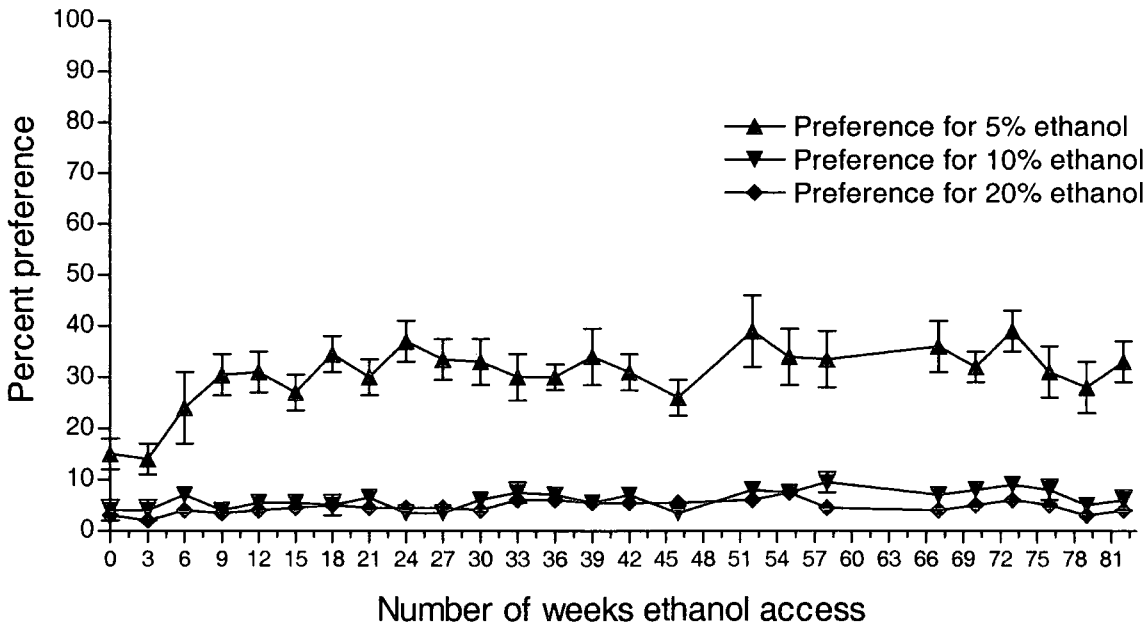


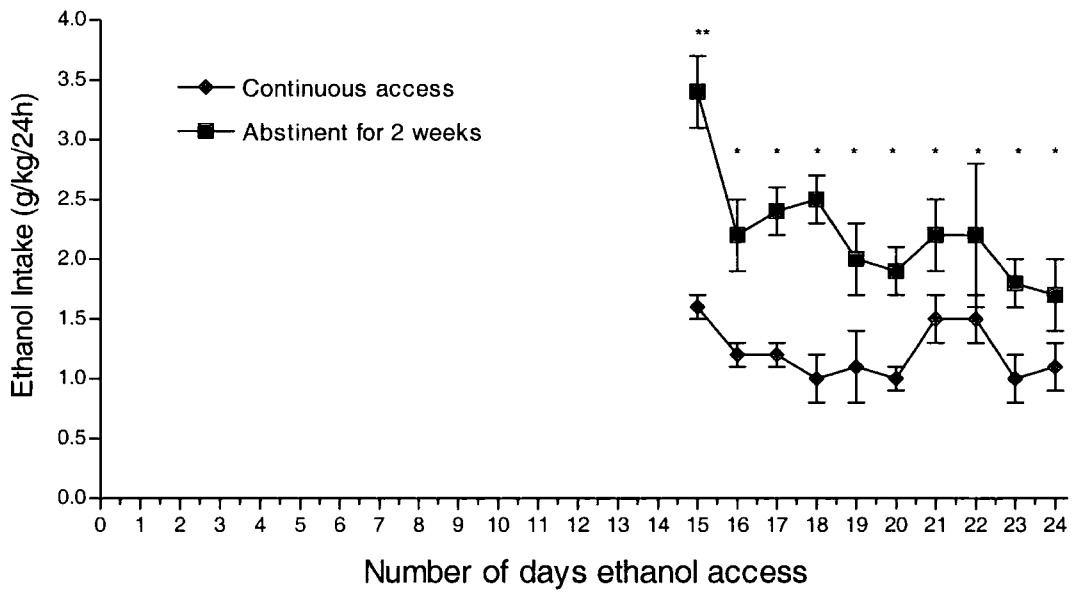
Figure 2.3: Wistar rats were housed in groups of four and given continuous free access to ethanol (5, 10, and 20% v/v) and water. After 55 weeks access to ethanol, 12 animals were withdrawn for 2 weeks and then reintroduced to ethanol (5, 10, and 20% v/v) and water (■). 12 control animals were provided with uninterrupted access to the four bottle choice of ethanol and water (◆). Data is presented as g of ethanol consumed / kg animal weight / 24 h.

* = $P<0.05$ for comparison with continuously drinking controls on the same day.

** = $P<0.005$ for comparison between the ethanol intake of the reintroduced group and continuous drinking controls on the same day.

† = $P<0.005$ for comparison between ethanol intake on day 15 with intake over the following 9 days (16-25).

Figure 2.3: The effect of 2 weeks abstinence on the ethanol intake of group housed Wistar rats with access to 5%, 10% and 20% ethanol (v/v) and tap water.



The effect of nimodipine on ethanol consumption

When this group of rats was transferred from housing in groups of 4 to single housing, they had an elevated ethanol consumption (3.22 ± 0.2 g/kg/24h) on the first day (Day 1) after the move (Figures 2.4 and 2.5), which then decreased sharply on Day 2 to 1.78 ± 0.1 g/kg/24h ($P < 0.001$, for comparison between consumption on Days 1 and 2) and remained stable near the lower intake level on Day 3 (2.0 ± 0.2 g/kg/24h).

The first injections of nimodipine, at 5 mg/kg or 20 mg/kg, on Day 10 of the single housing period, had no significant effect on either the ethanol intake of the animals with uninterrupted access to ethanol, iii, (Figure 2.4) or the total fluid consumption of any of the animals. During Days 18 to 23, the period when the "water" animals (Group i) were introduced to ethanol for the first time, and the "ethanol withdrawal" group (Group ii) were reintroduced to ethanol, there was no significant effect of nimodipine, at either 5 or 20 mg/kg, on the ethanol intake of these two groups when compared with the animals that were given Tween injections. There was also no effect of nimodipine on the "continuous drinking" group (Group B iii) that had access to the ethanol solutions throughout this period (figure 2.6).

There was, however, a significant effect of the ethanol access regimen on the ethanol consumption of animals on Days 18 - 23 (Figures 2.4, 2.5 and 2.6). Control animals (group i) drank significantly more ethanol than the group with continuous access to ethanol (group iii) over the 6 days of ethanol access ($P < 0.05$ for analysis of variance across all six days). During this same period (from Day 18 to 23), there was a significant difference between the ethanol intake of control animals (group i) and the abstinent group (group ii) on Days 19 ($P< 0.0005$), 20, 22 and 23 ($P < 0.005$). There was a significant difference between the ethanol intake of the animals

withdrawn and reintroduced to ethanol (group ii) and those with continuous access to ethanol (group iii) on the first day of reintroduction (day 18) ($P<0.01$). However, during the last five days of ethanol intake measurements, there was no significant difference between the ethanol intake of animals with continuous ethanol access (group iii) and that of the animals that remained abstinent for 2 weeks (group ii).

The animals that had continuous access to ethanol showed a significant increase in the variance of the group means during the injection period (Days 10 to 23, Figure 2.4). All the animals with continuous ethanol access had significantly increased variances during the injection period, Days 10-23, when these were compared with the pre-injection period, Days 1-9 (F-test results: Tween $F_{[45.70]}=3.78$ $P < 0.05$; 5 mg/kg nimodipine $F_{[50,82]}=6.97$ $P < 0.01$; 20 mg/kg nimodipine $F_{[45,69]}=1.59$ $P<0.05$, all comparisons being between the pre-injection and post-injection results for each treatment group)

Figure 2.4: Wistar rats were housed in groups of four and given access to ethanol (5, 10 and 20% v/v) and water for 55 weeks. Rats were then separated to individual housing with the same four bottle choice of ethanol and water. After 9 days baseline measurements of ethanol intake, ip injections of 5 or 20 mg/kg nimodipine or Tween vehicle were administered daily (days 10 to 23). Ethanol intake was measured daily throughout and is expressed here as the g of ethanol consumed / kg animals weight / 24 h. Tween vehicle (◆), n = 6. 5mg/kg nimodipine (■), n = 7. 20mg/kg nimodipine (▲), n = 6.

* = $P<0.001$ for comparison between the ethanol intake of all groups on day 1 with all groups on day 2 of ethanol access.

Figure 2.4: The effect of 5 and 20 mg/kg nimodipine (or Tween vehicle) on the ethanol consumption of Wistar rats with free access to 5%, 10% & 20% ethanol (v/v) and tap water. Injections started for day 10.

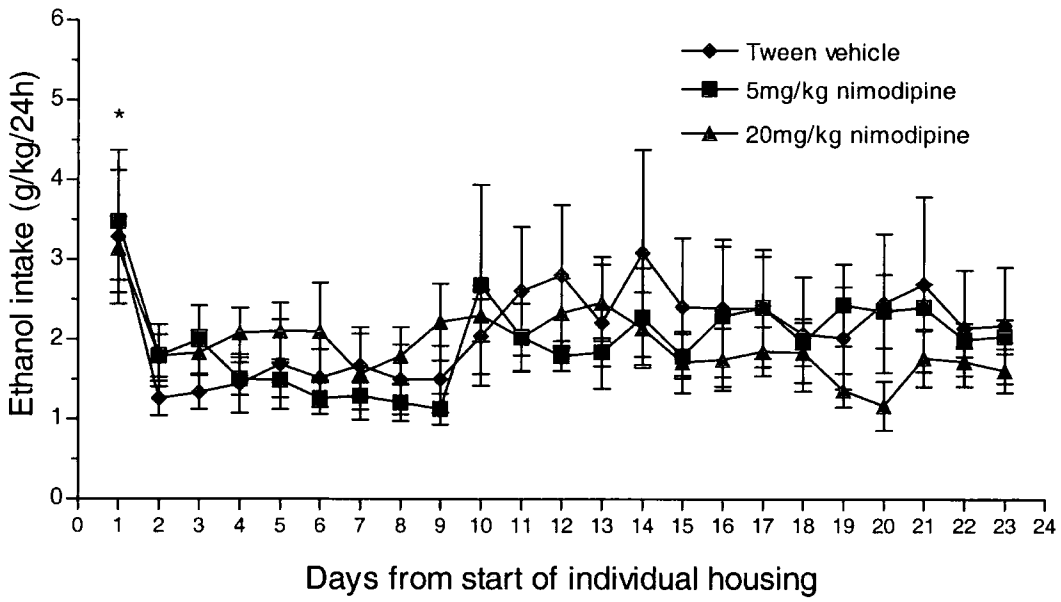


Figure 2.5: Wistar rats were housed in groups of four and given access to ethanol (5, 10 and 20% v/v) and water for 55 weeks. Rats were then separated to individual housing with the same four bottle choice of ethanol and water. After 3 days baseline measurements of ethanol intake, ethanol was removed from all cages for 2 weeks (days 4 to 17). I.p. injections of 5 or 20 mg/kg nimodipine or Tween vehicle were administered daily (days 10 to 23). Ethanol was reintroduced on day 18 (through to day 23) and ethanol intake was measured daily throughout and is expressed here as the g of ethanol consumed / kg animals weight / 24 h. Tween vehicle (◆), n = 7. 5mg/kg nimodipine (■), n = 7. 20mg/kg nimodipine (▲), n = 7.

* = $P<0.001$ for comparison between the ethanol intake of all groups on day 1 with all groups on day 2 of ethanol access.

Figure 2.5: The effect of 5 and 20 mg/kg nimodipine (or Tween vehicle) on the ethanol consumption of Wistar rats withdrawn for 2 weeks from access to 5%, 10% & 20% ethanol (v/v) on day 4 and reintroduced to ethanol on day 18.Injections started for day 10.

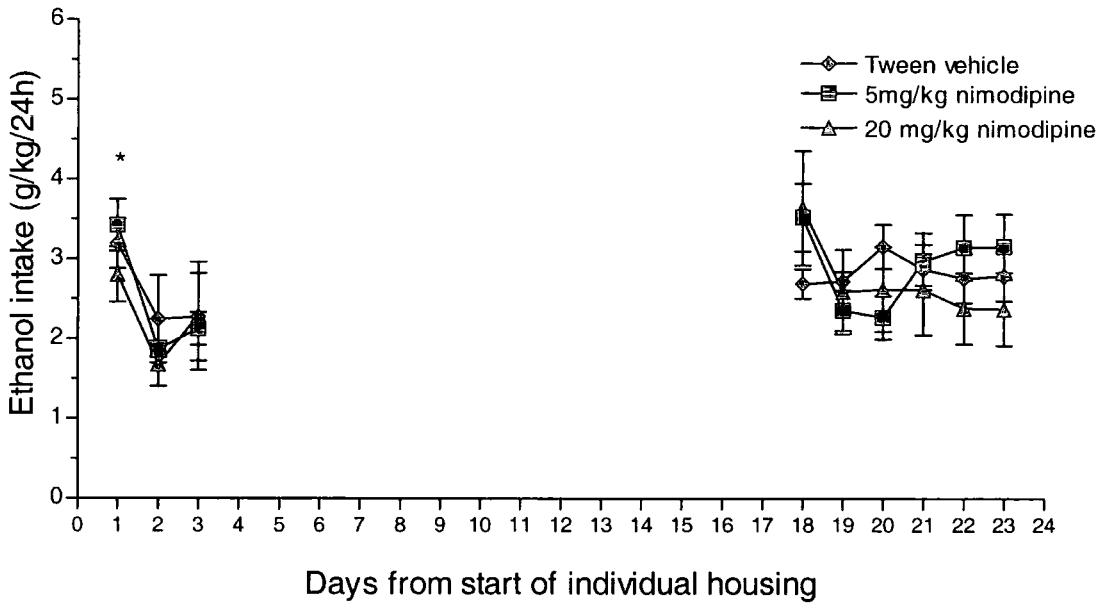
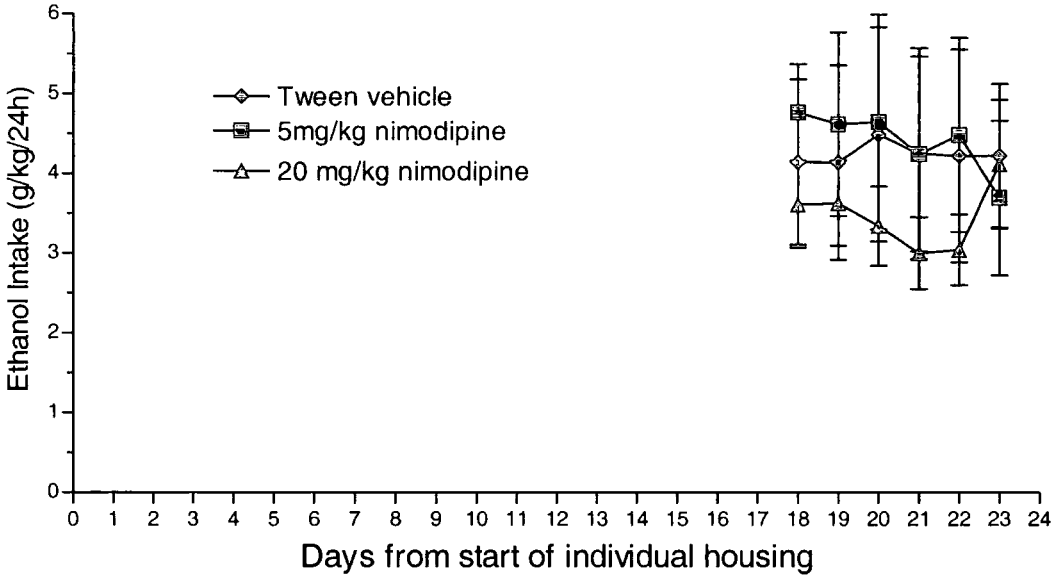


Figure 2.6: Age-matched control Wistar rats were housed in groups of four and given access to water only for 55 weeks. Rats were then separated to individual housing with continued access to water only. After 9 days 'baseline measurements', i.p. injections of 5 or 20 mg/kg nimodipine or Tween vehicle were administered daily (days 10 to 23). On day 18 (through day 23) rats were introduced to the four bottle choice of ethanol (5,10 and 20% v/v) and water. Ethanol intake was measured daily throughout and is expressed here as the g of ethanol consumed / kg animals weight / 24 h. Tween vehicle (◆), n = 6. 5mg/kg nimodipine (■), n = 7. 20mg/kg nimodipine (▲), n = 7.

Figure 2.6: Control Wistar rats introduced to ethanol after 1 week of nimodipine (5 and 20 mg/kg dailyor Tween vehicle). Injections started for day 10.



Discussion:

Models of ethanol consumption: the four bottle preference.

The results from both groups A and B clearly show that the four bottle choice method of alcohol access provides a long term ethanol consumption level of around 1.56 g/kg/day in our laboratory. The presence of the alcohol deprivation effect after 2 weeks abstinence from ethanol access in this model would indicate that the ~1.56 g/kg/24h ethanol consumption of animals with a choice of four bottles is sufficient ethanol intake for physiological effect and supports the idea that this model of ethanol intake has some degree of relevance to long term ethanol intake. This ethanol intake level is roughly equivalent to 34mM plasma ethanol assuming the ethanol was all consumed in one large binge, or 2.6mM per hour during a 12 hour awake period. Little *et al.*, (1999) indicates that plasma levels of ethanol up to 5mM produced alterations in mood in humans and 11mM was necessary for impaired attention. Thus, while these animals are possibly consuming sufficient ethanol for minor emotional changes, the intake is probably too low to produce any behavioural changes in rodents that we can observe or measure – Little *et al.* (1999) suggest 20mM as the lowest plasma concentration with measurable effects.

It is possible that the elevated ethanol intake after a period of abstinence was due to the novelty of the ethanol solutions being introduced (as with the control group). However, if this were the case then the ethanol intake would not be expected to remain elevated as the novelty wore off (as in the control group) and the novelty effect might be expected across all three ethanol concentrations for at least the first day of ethanol access. Thus it seems likely that the elevated intake is a true alcohol deprivation effect, although novelty can not been entirely eliminated.

Several of the key characteristics of human ethanol dependence were not seen when we used this model however. Wolffgramm and Heyne (1995) reported a steadily rising ethanol intake after 9 months of access and a switch in preferred ethanol concentration from 5% to 10%, yet neither of these effects was evident in either of the groups in this experiment. In fact, group A showed the opposite effect on ethanol intake with a slow decline in ethanol intake over 70-80 weeks. Wolffgramm and Heyne (1995) discussed the presence of a physical withdrawal syndrome after 9 months access to the three ethanol concentrations, although no data was presented in their 1995 article or any of the references provided. Following 55 weeks access to ethanol in this model, there was no indication of a physical withdrawal syndrome in our groups. This may simply be because the ethanol intake of the animals tested did not steadily rise, nor did their preferred ethanol concentration change and thus the individual ethanol intake was considerably lower than in the study of Wolffgramm and Heyne (1995). If our animals had consumed as much ethanol as those described by Wolffgramm and Heyne (1995) then a physical withdrawal syndrome may have been present. It should be noted, however, that passive observation of behaviours indicating a physical withdrawal syndrome (as used here) is not a very sensitive method for the measurement of ethanol withdrawal and so a low level withdrawal syndrome effecting heart rate and basal locomotion levels may have been present, although it is unlikely with this level of ethanol consumption (Holter *et al.*, 2000).

Other authors have attempted to use this model yet no one has published any results reproducing or opposing the findings of Wolffgramm and Heyne (1995). Spanagel *et al.* (1996) have reported an adapted four bottle choice model using monthly abstinence periods of three days, that causes a sustained, high level (~5 g/kg/24h) of ethanol intake in individually housed Wistar rats. Using the same

method of ethanol access and repeated abstinence, Hölter *et al.* (2000) reported a physical withdrawal syndrome only when measured using telemetric recordings of core temperature or locomotor activity. The crucial part to this adaptation of the study is the integration of the three day long abstinence period every month after the first 6 months of ethanol access (Spanagel *et al.*, 1996). An interesting point with respect to these two studies is also that they used Wistar rats from the Max Plank Institute of Biochemistry, Germany, whereas the Wistar rats used in my two studies were bred in Durham University from original stock supplied by Charles Rivers, UK. Dr Hölter has found that the animals supplied from the Max Plank Institute started out with a higher basal ethanol (~5g/kg/24h) intake than those from Charles Rivers (~basal intake of ~3g/kg/24 in the Hölter laboratory), and also that they responded more reliably to the repeated abstinence procedure (personal communication).

The role of voltage sensitive calcium channels in the modulation of ethanol intake in the four bottle preference model.

The administration of nimodipine had no effect on the ethanol intake of any of the access groups. When the data was re-examined for the effect of ethanol access regimen, irrespective of nimodipine or vehicle treatment, there was a significant elevation in the ethanol intake of abstinent rats reintroduced to the four bottle choice relative to that of the continuous access group ($P<0.01$). However, the level of ethanol intake of animals introduced to ethanol for the first time was also significantly higher than that of animals with continuous access to ethanol but not that of the abstinent group. This post-abstinence elevation in intake was to be expected in light of the data from the first study using the four bottle choice method of ethanol administration (Group A).

Nimodipine had no effect on the ethanol intake of Wistar rats with access to ethanol in three different concentrations. Whilst this is in contrast to the depressant effect of nimodipine on ethanol preference and intake reported in many previous studies (Smith *et al.*, 1999; Rezvani and Janowsky, 1990; Rezvani *et al.*, 1991; Fadda *et al.*, 1992; Pucilowski *et al.*, 1992, De Beun *et al.*, 1996a; Gardell *et al.*, 1997) the method of ethanol administration used here is unique among all of these earlier studies and the level of ethanol intake was at least half that produced in other studies using two bottle preference (Smith *et al.*, 1999; Rezvani and Janowsky, 1990; Rezvani *et al.*, 1991; Fadda *et al.*, 1992; Pucilowski *et al.*, 1992, De Beun *et al.*, 1996a; Gardell *et al.*, 1997) and this may be a critical factor in the effect of calcium channel blockade on ethanol preference.

One of the consequences of the protracted time period of the long term ethanol access model used in this experiment is that the animals are, inevitably, 15-18 months

old by the time they have spent in the order of 60-70 weeks drinking ethanol. Thus the lack of effect of nimodipine in the previous study may have been related to the age of the animals tested and not necessarily indicative of L-type calcium channels playing no role in the modulation of ethanol intake in this model.

The differences between the studies of Smith *et al.* (1999) are the species of animal used in the preference measurements (C57 mice v. Wistar rats) and the strain of rat used in the operant self-administration procedure (Sprague-Dawley rats v. Wistar rats), and also the method and level of ethanol administration. Both the C57 preference study and the operant self-administration provides a choice between two options (8% ethanol and water in the former and two levers in the latter; Smith *et al.*, 1999), whereas the four bottle choice model provides four options. There is a distinct possibility that a higher level of decision making is required with a choice between four options instead of just two and this greater cognitive processing may be controlled by 'higher' brain regions. In other words, the regulation of ethanol intake modulated by the various midbrain systems may be superseded by the cortical processing involved in the four choice decision making (Goel and Dolan., 2001).

In an attempt to address these questions two further studies were conducted using younger animals. Singly housed Wistar rats were given access to ethanol either in the 4 bottle choice method used with the older rats previously, or they were given access to ethanol in a more classical two bottle preference method providing a choice of 8% v/v ethanol or water.

The effect of nimodipine on ethanol intake of 'young' Wistar rats in a two or four bottle preference model.

Aim:

The aim of this study was to investigate whether calcium channel blockade by nimodipine has any effect on ethanol consumption of 'young' rats (less than 6 months old) when given access to ethanol as a choice between either 8% and water or between 5, 10 and 20% ethanol and water.

Methods:

Male wistar rats (150 - 200g, 7 weeks of age) were used in these studies. For each treatment group the n value was 7 in both experiments and the amount of fluid drunk from each bottle was recorded daily throughout the course of both experiments.

Experiment 1: 21 rats were singly housed with a four bottle choice of water, 5, 10 and 20% v/v ethanol. A baseline period of 6 days was allowed, then injections of nimodipine (5 or 20mg/kg) or tween vehicle were given once daily for the next four days, 1h before the start of the dark phase as before.

Experiment 2: 21 rats were singly housed with a two bottle choice of 8% v/v ethanol or water. A baseline of 7 days was allowed, then injections of nimodipine (5 or 20 mg/kg) or Tween vehicle were given once daily for the next three days, lh before the dark phase began as before.

The two bottle choice experiment was conducted providing ethanol as an 8% w/v solution rather than the 5% of the three bottle choice experiment because this is the standard concentration for two bottle preference methods, and the effects of dihydropyridines on ethanol preference previously reported used an 8% ethanol concentration (Smith *et al.*, 1999; De Beun *et al.*, 1996a, 1996b).

Animals in both experiments were housed in comparable laboratory conditions as the original study with long term ethanol access and nimodipine, i.e. 20°C, 60% humidity, large cages (60 x 38 x 20cm) with ad libitum access to laboratory chow.

Statistical Analysis

When results were compared over several days for two or more treatments, two way analysis of variance was used with one within-subjects factor (time) and one between subjects factor (treatment), with Student-Newman-Keuls as a post hoc test. Where comparison was made over more than one day, within a treatment group, one way analysis of variance was carried out, followed by Student-Newman-Keuls post hoc test. For comparisons between two treatment groups on a single day, or between two days for the same group, Student's t-test was used.

Prior to the start of the experiments it was decided that pairwise comparisons would be made only within the following treatment groups between the ethanol consumption on the day immediately preceding the first dose of nimodipine and the day of the first dose of nimodipine. This comparison was made for the continuously drinking animals receiving 5 mg/kg nimodipine, those receiving 20 mg/kg nimodipine and those receiving Tween vehicle. The F-test was used to compare the variances in the measurements of ethanol consumption. These comparisons were made for the ethanol consumption in each treatment group, at different times, as indicated in the Results section. Where the F test gave significant differences in the variance a Student's t-test would have been invalid and so pairwise comparisons were made using the non-parametric Mann-Whitney U test.

Results

Four bottle choice with young rats:

On the first day of ethanol access in the four bottle choice model, mean ethanol consumption was 3.8 +/- 0.3 g/kg/24h, however by the second day of ethanol access this consumption had dropped to 1.4 +/- 0.2 g/kg/24h which continued for the remainder of the baseline period (figure 2.7). When the nimodipine injections were commenced the ethanol intake increased significantly in all three treatment groups ($P< 0.05$ for comparisons between last intake before injections and first intake after start of injections). In addition, the variance of the consumption for each treatment group increased significantly ($P< 0.05$ for comparison between the last intake before injections started and the first intake after injections started). The intake remained at the higher level with increased variance for the remainder of the injection series and neither dose of nimodipine had any effect on ethanol intake. The total fluid intake did not change throughout the course of the experiment for any of the treatment groups.

Two bottle choice with young rats:

Rats given a choice of just two bottles, 8% v/v ethanol and water, had a mean ethanol intake of 3.4 +/- 0.5 g/kg/24h on the first day of ethanol access but this decreased to 0.5 +/- 0.3 g/kg/24h by the fourth day of the baseline period (figure 2.8). The baseline period was extended by one day in order to observe the consistency of the intake, which remained at the same low level. The administration of Tween vehicle or 5 or 20 mg/kg nimodipine had no effect on this low level of ethanol consumption at any time. Total fluid levels did not change throughout the course of the experiment for any of the treatment groups.

Figure 2.7: Singly housed Wistar rats had access to ethanol (5, 10 and 20% v/v) and water. Ethanol intake was measured daily for the first 6 days of access to provide baseline levels of intake. Nimodipine (5 or 20 mg/kg. ip) or Tween vehicle was injected daily from days 7 through 10,with daily measurements of ethanol intake continuing. Data is presented as g of ethanol consumed / kg animal weight / 24h. Tween vehicle (◆), n = 7. 5mg/kg nimodipine (■), n = 7. 20mg/kg nimodipine (▲), n = 7.

* = $P<0.05$ for F-test comparison of the variance on day 6 with that on day 7.

Figure 2.8: Singly housed Wistar rats had access to ethanol (8% v/v) and water. Ethanol intake was measured daily for the first 7 days of access to provide baseline levels of intake. Nimodipine (5 or 20 mg/kg. i.p.) or Tween vehicle was injected daily from days 8 through 10, with daily measurements of ethanol intake continuing. Data is presented as g of ethanol consumed / kg animal weight / 24h. Tween vehicle (◆), n = 7. 5mg/kg nimodipine (■), n = 7. 20mg/kg nimodipine (▲), n = 7.

Figure 2.7: The effect of nimodipine (5 or 20mg/kg or Tween vehicle) on the ethanol intake of male Wistar rats with free access to 5%, 10% and 20% ethanol (v/v) and tap water. Injections were from day 7 through day 10.

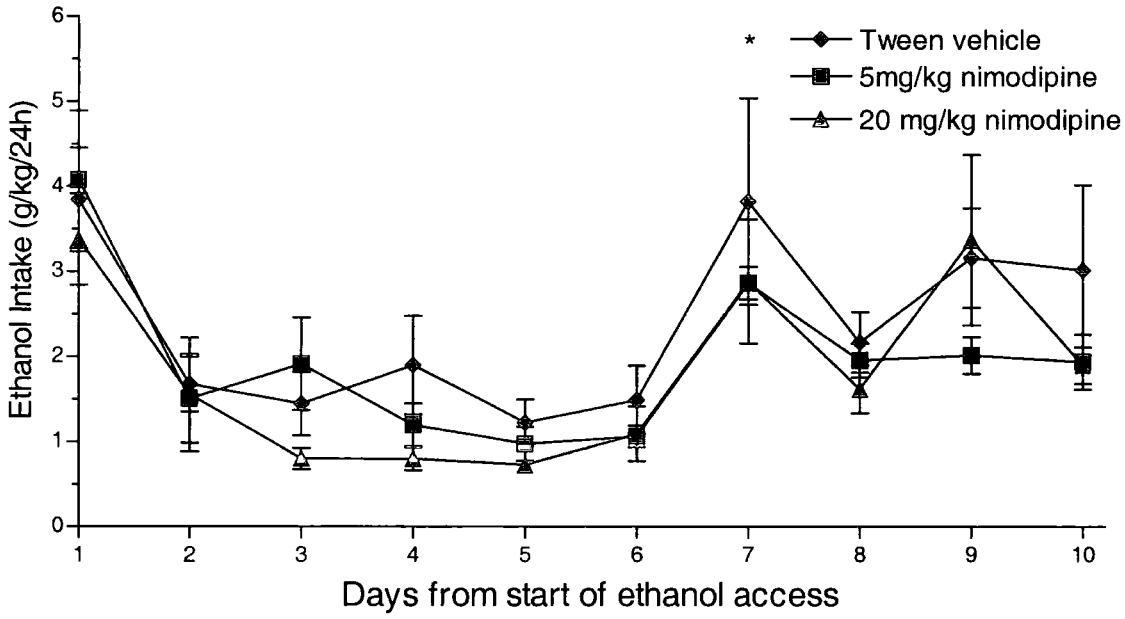
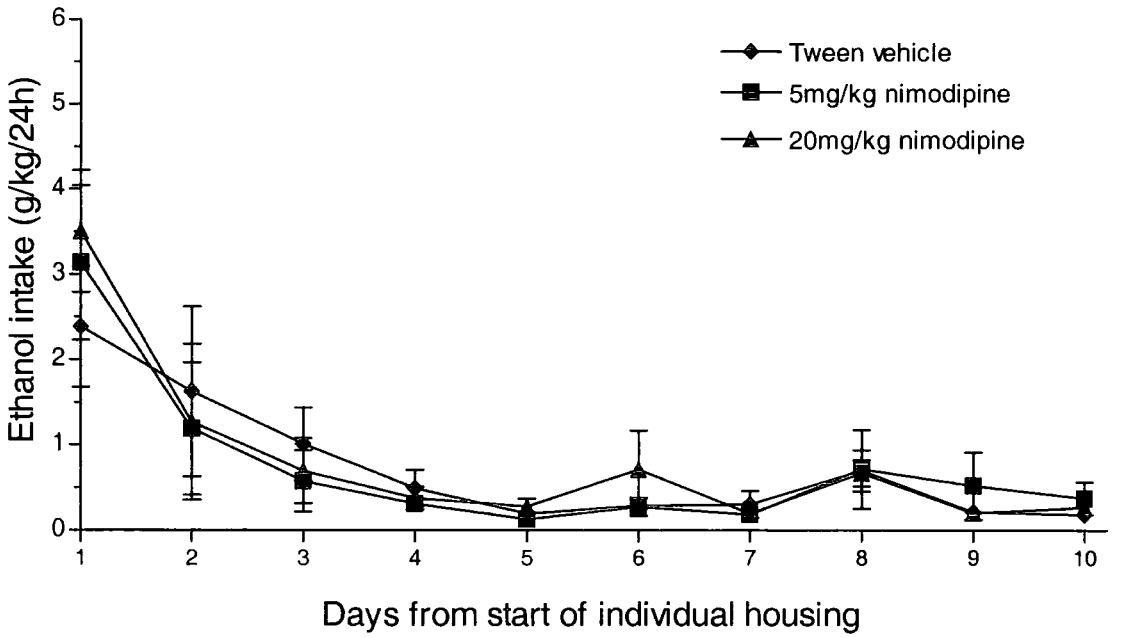


Figure 2.8: The effect of nimodipine (5 and 20 mg/kg or Tween vehicle) on ethanol intake of male Wistar rats with free access to 8% ethanol (v/v) and tap water. Injections were from day 7 through day 10.



Discussion:

Nimodipine did not seem to have any effect on the ethanol intake of animals given access to ethanol as a four bottle choice, although a small effect may have been disguised by the non-significant drift in the baseline. This result was evident irrespective of whether the animals had had 62 weeks experience with ethanol (and were subsequently 70 weeks old when nimodipine was tested) or whether they had had only 6 days experience with ethanol and were subsequently only 8 weeks old when exposed to nimodipine. These results demonstrate that the lack of effect of nimodipine in this model was not a result of the age of the test animals in the original long term ethanol access group.

If the increased number of choices in the four bottle preference model was of relevance to the lack of effect of nimodipine, then Wistar rats given a 2 bottle choice, would show a nimodipine-sensitive ethanol intake. However, as the data presented here shows, Wistar rats given a choice between 8% ethanol and water consumed very little ethanol (0.5 g/kg/24h) and nimodipine had no effect on this consumption. However, the volumes consumed in order to maintain an alcohol intake at this level are so small as to be very difficult to measure accurately and the loss of fluid through bottle movements or animals rubbing up against them provides a large percentage error relative to the small consumption. Thus the lack of effect of nimodipine seen here, may not necessarily be a true result since any decrease in ethanol intake would have been beyond the limits of detection by measurements of fluid intake with such a low baseline ethanol consumption.

Further manipulations were attempted in order to elevate the baseline intake of these animals prior to exposure to nimodipine such as:

1) Previous ethanol experience with four bottle preference to provide a high initial ethanol intake then switched to two bottle preference: ethanol intake dropped to almost undetectable levels (and the variance increased significantly) on switching to the two bottle choice.
2) Presentation of 20% sucrose and water and the gradual substitution of ethanol for sucrose (sucrose fading) until a two bottle choice between 8% ethanol and water was reached: animals consumed in excess of 30ml/day from the sucrose solutions but their intake gradually decreased to almost undetectable levels when they were presented with 8% ethanol and water alone.
3) Withdrawal of ethanol and reintroduction to two bottle choice after several weeks abstinence: no change in ethanol intake was observed after abstinence (intake remained at the limit of detectability). The lack of effect of abstinence was most probably due to the very low levels of pre-withdrawal ethanol intake.

No data has been presented for these studies investigating methods of elevating the ethanol intake of singly housed Wistar rats with a two bottle choice of ethanol or water because the experimental group sizes were small and no useful results were produced. Since none of the methods effectively elevated the ethanol intake to the range of accurate detection, nimodipine was not tested on these animals.

Comparison between the baseline period of the two methods of ethanol application (four bottle and two bottle choice) showed significant differences in the variances between the two methods of ethanol access ($P<0.01$, F-test) and a significantly higher ethanol intake of animals given a free choice between four bottles

over those choosing between two bottles ($P<0.01$, Mann-Whitney U test). This difference in intake has crucial significance in terms of the application of the models. Wistar rats given access to 8% ethanol and water, in our laboratory, did not consume sufficient fluid from the ethanol bottle to provide reliable measurements of ethanol intake. However, Wistar rats provided with a four bottle choice of 5, 10 or 20% ethanol and water consumed sufficient ethanol to measure accurately and investigate the alcohol deprivation effect. Thus it would seem that there is an important difference between the four bottle and two bottle methods of ethanol intake measurements, and the four bottle choice provides a higher level of ethanol intake.

One other factor to consider, however, is that in the four bottle choice ethanol is available at 5% v/v and this is the primary source of ethanol for all animals, whereas in the two bottle model, ethanol is available at 8% v/v. Whilst 8% ethanol has been widely used in two bottle preference models, the Wistar rats used here may have had a greater preference for 5% and found the 8% an unpalatable concentration of ethanol. My results give no indication as to whether this is true and a further investigation with Wistar rats given access to ethanol in a two bottle preference model with either 5% ethanol and water, or 8% ethanol and water, would be necessary to further clarify the relevance of the ethanol concentration available to this specific strain of rat in these models.

Chapter 3

Social status and ethanol intake

The influence of social status on individual ethanol consumption of group housed rats.

Introduction:

Animals living together in large populations in the wild, smaller populations in a large arena or burrow system, or a small group housed in laboratory cages develop a robust social structure (Dewsbury, 1982; Dunbar, 1988; Drews, 1993). Interactions within gregarious populations include various behaviours which can be classified as dominant or subordinate in nature. The original concept of the peck-order was introduced by Schjelderupp-Ebbe (1922) following observations of domestic fowl. With the introduction of the term dominance into the behavioural sciences, similar social structures were identified in other animals (Dewsbury, 1982; Gauthreaux, 1978). Following investigations and discussions of the strict definitions and displays of dominance (van Krefeld, 1970; Fedigan, 1982; Dunbar 1988; and Drews 1993), it became clear that the repetition of displays of dominance and the subsequent elicitation of subordinate responses in any given group of animals reflected a relatively stable dominance hierarchy or social rank.

The work of Syme (1974) highlighted several important factors required of a method of determining the social rank of group housed animals. Syme (1974) first classified ranking methods as either observational or competitive. The former are the classically naturalistic methods used by Schjelderupp-Ebbe (1922) when reporting the social organisation of domestic fowl into a 'peck-order'. Competitive methods, however, grew up from researchers manipulating the test environment in order to stimulate direct aggressive conflict between individuals for priority access to an approach situation (eg, food, water) or away from an avoidance situation (eg foot

shock). However, aggressive orders (i.e. orders in which rank is determined by the number of group mates an animal is aggressive towards rather than being attacked or threatened itself) stand outside this classification since their existence does not depend on an artificially restricted environment, yet the aggressive acts can be precipitated by social proximity (for example) and are not merely an observational measure.

The rationale behind the validity of competitive orders, such as the methods used today, as indices of dominance was (and is) that, since the dominance order governs all priorities in the group, it does not matter which behavioural rank is measured since they all represent the same phenomenon. This rationale, however, requires the validity of a unidimensional view of dominance. This unidimensional theory of dominance, however, has not been supported by research crossing several decades indicating that external factors such as reactions to the test environment as a group will alter the behaviour of individuals irrespective of their position in the social hierarchy (eg. Baeninger, 1970; Syme *et al.*, 1974; Gentsch *et al.*, 1988).

In order to avoid the question of the validity of a unidimensional theory of dominance, there are several methodological considerations involved with competitive ranking measures. For a competitive measure to be valid, the achievements of an individual in the test must not be influenced by an individual's ability to perform this particular task. This was termed the internal validity of a rank (Syme, 1974). Subsequent to the development of a test that is not merely a reflection of individual skill, the generality or external validity of the measure must be confirmed. That is to say that the competitive order should be shown to relate to other social behaviours and should not be response-specific.

Contemporary dominance studies primarily follow the definitions of Hand (1986). Dominant animals are characterised by aggressive and/or intimidating acts,

be they attacks, threats of attack or encroachment, or signals which might suggest that attack may occur (Hand, 1986). Subordinate rank is attributed to a consistent pattern of losses in certain conflict encounters (Hand, 1986). In any given encounter submissiveness is an active response to an aggressive action by another individual (Hanby, 1976) in which yielding or surrendering are displays given by a loser (Hand, 1986). Within a well established group the social hierarchy is maintained by continuous expression of dominant and subordinate characteristics by individuals in confirmation of their social status. This continuous dominance may not lead to fully agonistic encounters (aggressive fighting usually resulting in physical injury) but rather to low levels of aggression and threat which the subordinate animal responds to with submissive displays to avoid confrontation, wounding and defeat. This can result in the subordinate animal being under a continuous 'low' level of stress due to conflict avoidance. This is commonly referred to as a chronic social stress.

In a mixed-sex colony of rats in a visible burrow system the reinforcement of the social hierarchy can lead to sufficient stress to induce profound behavioural, physiological, and endocrine changes in the subordinate males (Blanchard et, 1995, 1992, 1993). Within the visible burrow system model, subordinate animals have been reported to show a great diversity of behavioural, physiological and neurochemical differences from the dominant animal (Table 3.1). The accumulation of these alterations in physiology and behaviour are thought to be the cause of early mortality compared with dominant males and individually housed controls (Blanchard et al, 1995, 1992, 1993).

At the other end of the social order, dominant animals show a greatly reduced neophobia compared with subordinate animals, indicated by higher locomotion levels in a novel arena, faster approach time to novelty (decreased latency of approach),

increased levels of exploration, and a longer time spent eating a novel food (Nott and Sibly, 1993). As described, dominant animals tend to utilise aggressive displays which elicit submissive actions from subdominant and subordinate animals. These aggressive displays can be readily seen when in direct competition for pleasant food or liquids, whilst subordinate animals will be slow to approach and reluctant to compete (Gentsch *et al.*, 1990; Nott and Sibly, 1993; Brain and Benton, 1983; Bartos *et al.*, 1994; Blanchard *et al.*, 1993).

Table 3.1: Changes in physiology, neurochemistry and behaviour of subordinate rats compared with dominant rats.

Physiological changes	**Reference**
Loss of body weight Increased adrenal weight Decreased thymus weight Increased plasma corticosterone levels Decreased plasma testosterone levels	Blanchard *et al.* 1995
Decreased testes weight Increased spleen weight Increased corticosterone levels but no change in aldosterone Decreased corticosterone binding globulin	Blanchard *et al.* 1993
Blood pressure oscillations	Fokkema *et al.* 1986
Increased heart rate Increased core temperature	Miczek *et al.* 1991
Neurochemical Changes	
5-HIAA (but not 5-HT) increased in : preoptic area amygdala hippocampus spinal cord entorhinal cortex Ratio of 5-HIAA/5-HT increased in : Hypothalamus Midbrain spinal cord DOPAC, Dopamine and DOPAC/DA ratio: No change	Blanchard *et al.*, 1991
Increased tyrosine hydroxylase mRNA in locus coeruleus Increase tyrosine hydroxylase protein levels in locus coeruleus	Watanabe *et al.* 1995
Increased Pro-opiomelanocorticotrophin mRNA in the pituitary gland No change in corticotrophin releasing hormone in the hypothalamus No change in corticosterone (type I or type II) receptor mRNA in the hippocampus	Blanchard *et al.* 1993
Behavioural Changes	
Increased neophobia: - increased latency of approach to novelty - decreased open field locomotion - decreased feeding time	Nott and Sibly, 1993
Fewer episodes of : - eating and drinking - social contacts - mounts of females - transits from one housing area to another	Blanchard *et al.* 1993
Decreased locomotion in the open field Decreased rears in the open field	Raab *et al.* 1986

The role of social status in ethanol intake:

A key factor in human alcohol consumption and addiction is that of psychosocial stress. Stressful life events can precipitate drinking spells, heavier than normal drinking, and relapse after abstinence. Chronic 'social stress' from 'life pressures' can result in relapse and heavy drinking (see thesis introduction). Several studies using animals have shown that social status, such as subordinate or dominant rank, can produce higher or lower levels of alcohol intake respectively (Blanchard *et al.*, 1992; Ellison, 1987) whilst both chronic and acute social stress increase levels of ethanol consumption (Wolffgramm and Heyne, 1991; Wolffgramm, 1990). The studies of Ellison (1987) and Blanchard *et al.* (1992) both used large, sophisticated, test arenas, providing an enriched environment for groups to build up a complex social hierarchy. However, as discussed in the previous chapter, the system of chronic ethanol consumption of Wolffgramm and Heyne (1995) used animals in standard cages and in groups of four. The ranking of animals in this controlled laboratory environment has been carried out quite extensively using competition for food, exploration behaviours and locomotion levels as relative measures of dominance, as well as direct measures of aggressive and submissive behaviours in established and novel social groups. However, there is very little information about the social hierarchy of animals housed in groups of four in standard laboratory conditions and there has been no investigation into the correlation between the ethanol intake of individual animals group housed in this manner and given access to three concentrations of ethanol and tap water as in the model described in the previous chapter.

Ranking measures: established and new methodologies

Ranking measures used over the last decade (Gentsch *et al.*, 1990; Nott and Sibly,1993; Woodall, 1996; Mitchell and Redfern, 1992) have centred around simple

characteristics of either the dominant or subordinate animal. Following the idea that the dominant animal is larger, more aggressive and essentially 'greedier', fighting for and winning the greater amount of access to rewarding food and stimuli, Gentsch *et al.*, (1988, 1990) used competition for a sucrose pellet, with scores being allocated for: approach; contest; and winning the pellet. Other authors (Joly and Sanger, 1991; Joly and Sanger, 1992; Woodall *et al.*, 1996) have used competition for a limited amount of sweetened milk, with rankings obtained from the number of 5 second time bins spent drinking.

Alternative methods have used the neophobia of the subordinate animal. This resistance to investigate novelty most probably arises from repeated defeat and a subsequent avoidance of any confrontation. Nott and Sibly (1993) have shown dominant rats display a decreased latency of approach to novel food in a familiar open field arena, whilst subordinate animals will be reluctant to investigate the food.

The most direct method used for ranking, however, is based around the premise that the subordinate animal will go to great lengths to avoid investigating or threatening any other group member, whilst the dominant animal will exert its dominance through repeatedly investigating, aggressively grooming and attacking its cage mates. Simple observation of aggressive interactions either within the home cage or open field and scoring individuals based on aggressive and submissive acts can give a good indication of the relative position of individuals within their home group hierarchy (Mitchell and Redfern, 1992; Bartos *et al.*, 1994; Wolffgramm and Heyne, 1990). However, there has been some discussion over whether this is a true demonstration of a social rank and not just an 'aggressive rank' that is not generalisible to other social behaviours of a dominant animal (Syme, 1974, Brain and Benton, 1983).

While these methods provide robust information about the position of any given individual in their competitive order, it is clear that each experiment uses different characteristics of the dominant or subordinate animal. Indeed, it would seem particularly relevant to behavioural studies that the ranking methods used provide either:

a) a universal ranking order irrespective of ranking method used, or

b) a rank based upon a behavioural characteristic pertinent to the behavioural test being used and the disorder examined.

A major consideration in the selection of ranking measures for this study was the over-riding need for tests that elicited a clear display of the behaviour being tested, without further elevating the stress levels of individuals. The tests previously discussed all used repeated testing with a limited goal resource, and incurred a level of disruption to the daily routine and rhythm of the test groups. This disruption may have in itself elevated the stress levels of the test animals and therefore artificially elevated alcohol intake, hiding any effect of the social status and subordination stress on individual ethanol intake. In order to avoid this disruption published methodologies (Nott and Sibly, 1993; Gentsch *et al.*, 1988, 1990; Joly and Sanger, 1991, 1992; Woodall *et al.*, 1996; Mitchell and Redfern, 1992; Bartos *et al.*, 1994) were re-designed with the specific goal of minimising experimental changes in individual stress levels and ethanol intake, whilst eliciting behavioural responses that could be interpreted and indicative of social status.

Three basic ranking methodologies were chosen for this study. The methods were chosen to cover three different behavioural aspects of the dominant and subordinate status while also allowing for controlled limitation of stress input from the experimental procedures. The three methods chosen were:

1) Competition for a rewarding food

In the studies of Gentsch *et al.* (1988, 1990), Joly and Sanger (1991, 1992) and Woodall *et al.*(1996) discussed previously, a limited amount of sucrose was provided in order to elevate the level of competition. However, this required a repetition of use of the method in order for all the animals to realise that the sucrose was available and to compete. Our method provided a 20% sucrose solution in a standard drinking bottle with no other fluid available. The test time was sufficiently long enough that all the animals were aware of the presence of the sucrose and the essentially unlimited supply of sucrose solution ensured that there was a continuous competition between all animals rather than limited confrontation over a single reward.

2) Time taken to approach a novel item or food in an familiar open field

Nott and Sibly (1993) have shown that dominant rats display a shorter latency of approach to novel food in a familiar open field arena than subordinate animals who were reluctant to investigate the food. A common manipulation of these open field behaviours is to use the open field as an arena for confrontation and avoidance behaviour whilst competing for novelty (usually food), marking the dominant and subordinate animals respectively. Our measure, however, attempted to decrease the stress levels involved in aggressive confrontation in a novel, exposed arena by habituating animals to the open field individually and then presenting a small amount of novel food in order to precipitate the subordinate expression of neophobia without inducing large elevations in the anxiety-related stress levels of individuals.

3) Measurements of aggression and submission in direct social interactions.

This is the most direct method for ranking animals and was based on the premise that the subordinate animal would go to great lengths to avoid investigating

or threatening other group members, whilst the dominant animal would exert its dominance through repeatedly investigating, aggressively grooming and attacking it's cage mates. Simple observation of aggressive interactions either within the home cage or open field and allocation of the social status of individuals based on aggressive and submissive behaviours provides a good index of the relative position of individuals within their home group hierarchy (Mitchell and Redfern, 1992; Bartos *et al.*, 1994; Wolffgramm and Heyne, 1990). This method is more commonly used to rank animals on their first meeting, when aggression levels are high and the social hierarchy is first fought out. However, our method used the period of investigation between animals from an already established hierarchy that had been separated for 3 hours. The re-exertion of the social hierarchy under these conditions did not result in overt aggressive behaviour and fighting which would have artificially elevated stress levels, but sufficient social investigation and aggressive posturing was observed to provide an index of social status within each group of cage mates.

Aims:

The aim of this study was to examine whether less disruptive (and so less stressful), single test, ranking methodologies based around three different paradigms

1) measurement of success in competition for a food reward;
2) measurement of neophobia in an open field arena;
3) measurement of aggression and submission in direct social interaction;

would provide useful competitive ranks for the prediction of individual ethanol intake of male rats housed in groups of four.

Methods:

Animals

Male Wistar rats (mean starting weight 194 ± 1.3g) were housed in groups of four in standard rat cages measuring 60 x 38 x 20cm. They were housed on a normal phase light cycle (12h/12h light/dark, lights on at 7am), with room temperature maintained at 21°C, and the humidity between 40 and 60%. Standard laboratory chow was available *ad libitum*. In 3 cases, animals were removed from the study before the start of the behavioural assessments because they showed an unexplained loss of body weight.

Ethanol Access

Chronic ethanol intake while group housed

Group housed rats were given continuous free choice access to four bottles containing tap water, and 5, 10 or 20% v/v ethanol for 82 weeks. Every three weeks the mean 24h ethanol and total fluid consumption was determined by measurements on three consecutive days, providing 2 sets of 24h consumption values. The results were expressed as g/kg/24h and the mean of two 24h measurement periods for each cage was used to calculate the mean value across cages. Control animals were given access to four bottles all containing tap water.

Ethanol intake of individual animals in a group housed environment

In order to determine the ethanol preference of individual animals, each group was placed in a standard home cage with a modified Perspex lid to allow their activity to be video-taped. The animals were first weighed and marked with Indian ink as described in the following section (page 157). After 24 hours of habituation to the new cage and test room, the animals were recorded for the first 8 hours of their dark

phase. Individuals were visually scored for time spent licking each bottle neck as an index of drinking time and thus volume consumed relative to cage mates. The weight change in the bottles during the test period was recorded. Individual drinking time at any given bottle was converted to a fraction of total time spent at that bottle by the group. This (along with the total weight change of the bottle) was then used to determine the amount of each solution consumed by each individual animal. Observations were made over the first hour of the dark phase to cover the period of highest activity, and for 30 minutes at 2, 4 and 6 hours from the change of light phase to ensure any delayed drinking by subordinate animals was not missed.

Test Groups

Six cages (total 24 animals) were given free access to tap water, 5, 10 and 20 % v/v ethanol, while 6 cages (total 24 animals) were given free access to four bottles of tap water only. Animals were left with continuous access to ethanol (or water in the case of the age-matched controls) for 82 weeks and mean ethanol intake for each cage (containing four individuals) was measured for the entire period.

After 82 weeks access to ethanol or water, individuals were allocated positions within their home cage hierarchy as described below. Following determination of social rank by all three methods, the ethanol intake of individual animals was measured as described above. Control animals were introduced to the three concentrations of ethanol and their individual ethanol intake on the first and 10^{th} day of ethanol experience was measured. Between the 1^{st} and 10^{th} day of ethanol access, 1 animal died and the results from 1 cage of animals was lost on the 10^{th} day of ethanol access due to a technical fault in the video-recording system.

Behavioural Observations and Scoring

Except for the open field experiment, all observations were made with the animals housed in a standard cage (60cm x 38cm x 20cm) with a modified Perspex lid to provide an unobstructed view (test cage). All behaviours were observed under red lights and were recorded using a black and white low-light camera and domestic video recorder. All groups were habituated to the test cage and room for 24 hours prior to testing. Animals were marked with indian ink to aid identification during testing. Marking was standardised to: no markings, shoulders only, hind quarters only, spinal stripe.

All behavioural observations were scored by a single observer and recorded using the Observer PC software. Individuals were allocated a rank from 1 - 4 (1 being dominant) for each ranking experiment.

Determination of Social Rank

Competition for Sucrose solution

Animals were placed in their original home cage groups, into a test cage as described above to allow for video-recording of the time each animal spent drinking the sucrose solution. Following the 24h habituation period, at the beginning of the dark phase, all food and water was replaced with a single bottle of 20% sucrose solution. The competition and access for this solution was recorded for 30 minutes before the solution was removed and the food and water returned. To ensure continuous competition throughout the test period sufficient sucrose solution was provided so that the bottle was not emptied during the course of the experiment. Time spent drinking was recorded relative to the rest of the group and the total volume of sucrose consumed was measured. The amount of time each individual spent drinking was divided by the total time the entire group spent drinking. This gave the

proportion of the total sucrose consumed by each individual and was expressed as grams of sucrose per individual. Dominant status was awarded to the animal drinking the most sucrose, whilst animals that drank the same amount were allocated the mean score from their pooled positions.

Approach latency to novel food in the open field

During the light phase (but under red light conditions) individuals from one home cage group were placed in the testing room and were separated to four individual cages and given access to laboratory chow and water only. Isolated animals were not provided with alcohol since Wolffgramm and Heyne (1991) demonstrated that the transition from group housed four bottle choice ethanol access to individual housing caused a significant increase in the ethanol intake of individuals. Subjects were individually housed in this manner for 20 minutes in order to provide uniform stress levels for all animals before testing. Individuals were then introduced, in isolation, to a novel open field arena (100 cm x 100 cm x 50cm) with a central stage. After a 30 minute period of habituation to the open field arena, a small amount (~5g) of novel food (guinea pig food or sunflower seeds - *high street pet shop*) was placed on the central stage. The latency to approach the food was recorded for each individual. Approach was considered complete when the subject placed both front paws on the central table top. Two different novel foods were made available in a randomised manner (i.e. each individual only saw one food type but which type was randomly allocated) in order to control for the influence of individual taste/scent preferences for the food provided.

A maximum cut off time of 20 minutes was applied since all animals examined and crossed the central stage in the 30 minute habituation period so could

be expected to approach again if undisturbed. After a 20 minute period without investigation 'No Approach' was scored. The test period from habituation to approach to the novel food was video-taped and if more than 2 animals did not complete the approach behaviour in 20 minutes then their rankings were based on the level of investigation of the stage and food, with the dominant animal investigating more than the subordinate animal. After exposure to the open field arena, individual animals were returned to their individual cages until all four cage mates had been exposed to the open field arena. On completion of this experiment, cage mates were reintroduced to each other in their original home cage, the behavioural interaction involved was used in the next method of this study.

Reintroduction to established cage mates

At the end of the open field experiment which resulted in a total of 3 hours of separation, the cage mates were returned to the test home cage and videotaped for 1 hour. The separation period allowed for a higher level of social investigation and reassertion of rank than would occur in the undisturbed home cage. All interactions were observed and scored on the basis of:

Aggressive:

Investigator by anogenital sniffing

Any other aggressive behaviours

eg. sideways approach

attack resulting in supine posture of defeated animal

mutual rearing (recipient being the first to turn and / or walk away)

aggressive grooming

Submissive:

Recipient of investigation

Recipient of other aggressive interaction

Owing to the protracted social proximity of the groups prior to the test day few interactions occurred, the scores were expressed as a ratio of the number of instances of aggressive or dominant behaviour displayed by an individual over the number of submissive acts displayed or dominant acts received.

Statistical Analysis

The variance of the ethanol intake (mean group housed and individual intake) was compared using an F-test. Following confirmation that there was no significant difference in the variance of ethanol intake measurements, the long term, group housed, ethanol measurements for the 6^{th} week and 82^{nd} week of ethanol access were compared using a Student's t-test. Individual ethanol intake was compared between ethanol experienced and control animals using Student's t-test.

In order to compare the ranks allocated by each test and the rank ethanol intake for individuals, the hierarchical positions allocated from each test were compared using Spearman's rank correlation. Each position in the social hierarchy was given a value from 1 (dominant) to 4 (subordinate) in each test (Table3.2). These values were then correlated in a 'pairwise' manner.

Results:

Long term, mean ethanol consumption of group housed animals

These results are also presented in Chapter 2, Group A. Animals consumed 1.56 ± 0.05 g/kg/24h throughout the course of their ethanol experience.

Individual ethanol consumption

Individual ethanol intake ranged from as high as 6.0 g/kg/24 hours to no consumption at all in the alcohol experienced group (mean± sem = 2.73 ± 0.42 g/kg/24h). Preference ratios for individual alcohol concentrations ranged greatly. Preference ratios for the 5% alcohol solution were from 0 to 1.0, whilst the ratio for the 10% solution was from 0 to 0.83 and the preference ratio for 20% alcohol was from 0 to only 0.48. Only one animal consumed just one concentration of ethanol (5% ethanol) and no water but it consumed only 0.86 g/kg in 24 hours. Several animals consumed none of one or other of the alcohol concentrations. 11 of the 23 animals chose not to drink from the 10% bottle.

When age-matched control (ethanol naïve) animals were introduced to ethanol all subjects consumed some alcohol (mean±sem = 3.34±0.4 g/kg/24h). The intake ranged from 1.36 to 9.13 g/kg in 24 hours. Preference ratios for 5% alcohol ranged from 0.01 to 0.72. 10% alcohol preference ratios ranged from 0.01 to 0.42. All animals drank from both these concentrations, whereas the 20% alcohol solution was drunk with preference values ranging from 0 to 0.43. Only 2 animals of the 21 tested chose not to drink from the 20% bottle. The level of ethanol intake of ethanol naïve controls was not significantly different from that for the experienced animals and neither was the group variance (figure 3.1).

The same age-matched control group was monitored for individual consumption a second time 10 days after introduction to ethanol. The alcohol intake

of the control group was significantly decreased ($P<0.005$) compared to the first day of ethanol access, and now ranged from 0.15 to 5.07 g/kg/24h (mean ± sem = 1.55 ± 0.37 g/kg/24h; figure 3.1). The preference ratio for 5% alcohol solutions was from 0 to 1.0, only one animal consumed purely 5% alcohol and this amounted to just 0.11 g/kg in 24 hours. The 10% preference ratio ranged from 0 to 0.55, whilst the 20% preference ratio range was 0 to 1.0, with one animal consuming only 20% ethanol up to 1.46 g/kg in 24 hours. One animal in this group consumed from all three alcohol concentrations but not from the water bottle, totalling just 0.3 g/kg in 24 hours.

Figures 3.2 a,b, and c show the frequency distribution of individual ethanol intake for ethanol-experienced animals, and control animals on days 1 and 10 of ethanol access. These figures depict that, despite similar variance of ethanol intake, there was a distinct difference in the distribution of ethanol intake levels between groups. It is clear that a greater number of animals consumed larger levels of ethanol on their first day's access to ethanol as compared to on day 10. Figures 3.2b and c show the individual ethanol intake for the control group on the first and tenth days of ethanol access respectively. Chi squared analysis of the distribution of individual ethanol intake within each test condition showed a significant difference between all three distributions ($P< 0.01$ for all comparisons). Individual ethanol intake is thus very sensitive to the length of experience with ethanol. The apparent leftward shift of the long term (86 weeks) ethanol access group (figure 3.2a) represents a significantly higher number of individuals consuming low (< 2g/kg/24h) volumes of ethanol than control animals on the first day of ethanol access. This leftward shift is also seen after 10 days experience with ethanol (figure 3.2c) and the distribution of individual ethanol intake is significantly different between control animals on this day (day ten) and the first day of ethanol access (figure 3.2b).

Figure 3.1: Wistar rats housed in groups of four were given access to ethanol (5,10and 20% v/v) and water. The mean individual ethanol intake of animals with 82 weeks experience of ethanol access in this manner (Ethanol experienced) is presented alongside that of age – matched control animals on their first and tenth day of access to ethanol (5,10, and 20% v/v) and water after 82 weeks parallel experience with just water. Data is presented as g of ethanol consumed / kg animal weight / 24h. n = 24 for all groups.

** = $P<0.005$ for comparison between ethanol intake of control animals on day 10 and day 1.

Figure 3.1: Mean individual ethanol intake of group housed Wistar rats with either 82 weeks experience of ethanol access or on days 1 and 10 of introduction to ethanol.

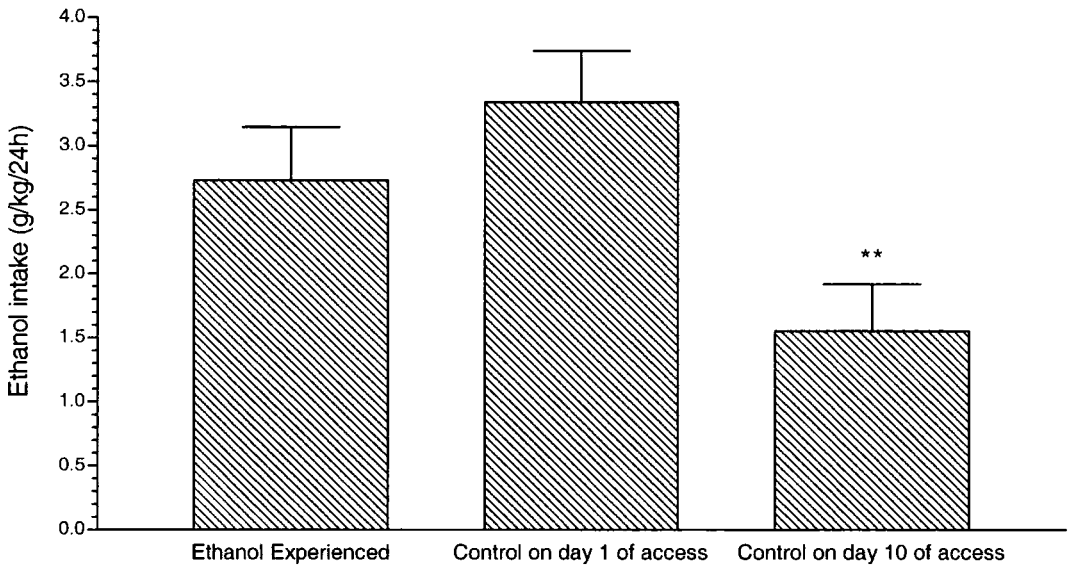


Figure 3.2a: Wistar rats were group housed with access to ethanol (5,10 and 20% v/v) and water for 82 weeks. Individual ethanol intake was determined after the 82nd week of access. Data presented here is the frequency distribution of ethanol intake (g of ethanol consumed / kg body weight / 24h).

Figure 3.2b: Wistar rats were group housed with access to water only for 82 weeks as parallel controls. These animals were introduced to ethanol (5,10 and 20% v/v) and water and individual ethanol intake was measured on the first day of ethanol access. Data presented is the frequency distribution of ethanol intake on the first day of ethanol access (g of ethanolconsumed / kg body weight / 24h).

Figure 3.2c: Wistar rats were group housed with access to water only for 82 weeks as parallel controls. These animals were introduced to ethanol (5,10 and 20% v/v) and water and individual ethanol intake was measured on the first and tenth day of ethanol access. Data presented is the frequency distribution of ethanol intake on the tenth day of ethanol access (g of ethanolconsumed / kg body weight / 24h).

Figure 3.2a: Frequency distribution of individual ethanol intake of male Wistar rats with 86 weeks experience of ethanol in a four bottle choice model.

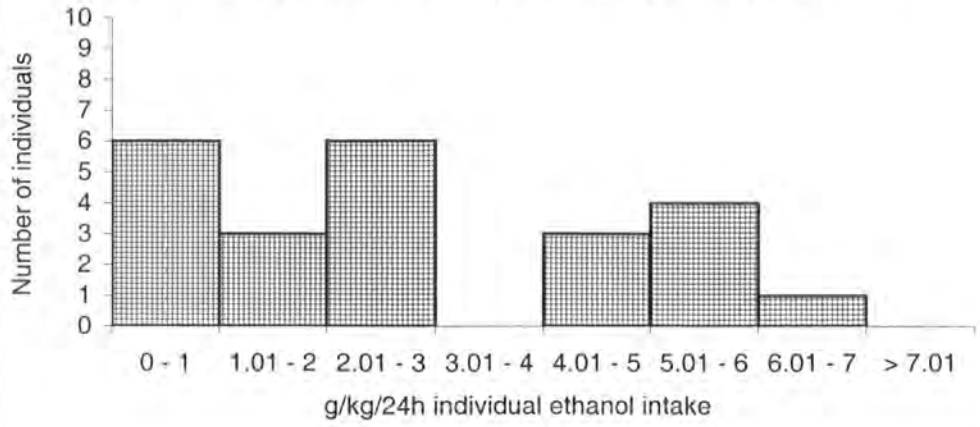


Figure 3.2b: Frequency distribution of individual ethanol intake of male Wistar rats on the first day of introduction to ethanol in a four bottle choice model.

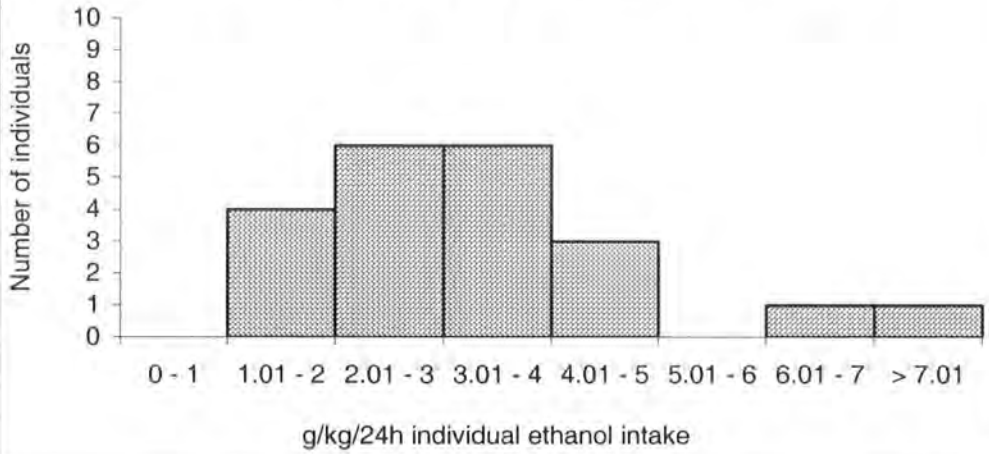
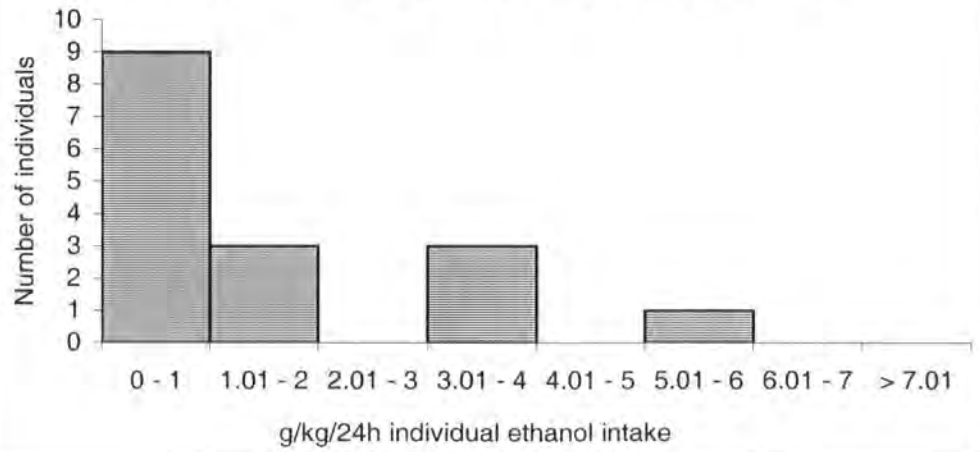


Figure 3.2c: Frequency distribution of individual ethanol intake of male Wistar rats 10 days after introduction to a four bottle choice model.



Social Rankings

Competition for Sucrose solution

During the test period all animals in all cages gained access to the sucrose solution. On two occasions combined access of 2 or more individuals at the bottle neck resulted in brief but direct conflict usually resulting in a single individual drinking with others attempting to displace him from the bottle neck. Dominance was measured by the time spent drinking the sucrose and the consequences of 'winning' these direct confrontations at the bottle neck was access to the sucrose solution. Thus any additional relevance applied to the conflicts at the bottle neck would be recorded twice – once as a victory in conflict and then again as a period of access to the sucrose (the direct consequence of the victory). In light of this fact, and the low occurrence of the conflicts at the bottle neck, no further analysis of these confrontations was included in this study. The time spent drinking by each individual animal ranged from 9.2 to 592s (mean ± sem = 324.1 ± 17.7s). Individual sucrose consumption was equally variable ranging from 1.2 g/kg to 84.6 g/kg (mean ± sem = 41.6 ± 2.9 g/kg).

The animals with 82 weeks experience of ethanol showed no significant correlation between their individual sucrose consumption levels and their ethanol consumption. However, a correlation was seen between rank preference for the 10% ethanol solution and sucrose consumption relative to co-habitants ($p < 0.05$, $r = 0.52$), that is the animals ranked highest (score of 1) by this method drank the most 10% ethanol (ranked 1) and the low ranked animals (score 4) drank the least 10% ethanol (ranked 4). Interestingly, there was a similarly correlation between sucrose consumption and rank g/kg/24h ethanol intake of age matched control animals on the first day of exposure to the three ethanol concentrations ($p < 0.05$, $r = 0.549$), this correlation was not seen ten days later however. There was also a correlation between

the sucrose consumption and rank order of consumption of 5% ethanol by control animals on the 1st ($p < 0.05$, $r = 0.461$) but not the 10th day of ethanol access.

Approach latency to novel food in the open field

Approach latencies varied from 15s to 'No Approach' within the 20 minute test period. However, all animals fully investigated the central table during the habituation period, and 4 animals showed signs of investigation during the test period but did not meet the approach criteria of placing both front paws on the table simultaneously. These animals were ranked higher than cage mates that scored 'No Approach' but lower than all animals that met the approach criterion

There was no significant correlation between approach latency and individual ethanol consumption of either the ethanol experienced or the ethanol naïve animals.

Reintroduction to established cage mates

On reintroduction to established cage mates, the level of social investigation and aggression was not particularly high. However, all animals except one were involved in social interactions as either the aggressor or the recipient at least twice. One particular cage showed a low overall level of investigation and dominance displays, and it was in this cage that one subject was not involved in any investigations or attacks. The index of aggression over submission ranged from 7.0 to 0, with 1 animal scoring 7.0 and 3 scoring 5.0, but only one scoring 0. Four animals received no investigation at all and were only involved in dominating acts. 12 animals had an index of less than 1, therefore receiving more investigations and dominant acts than they carried out, whilst 9 animals displayed more dominant acts than they

received (index greater than 1). The mean index for the entire group (± sem) was 1.45 ± 0.23.

No significant correlation was found between the level of investigation and individual ethanol consumption of either the ethanol experienced or the ethanol naïve groups.

Comparison of social ranking methods in control animals only

The rank allocated to each individual in each test is shown in Table 3.2. As can be readily seen from this data, there was no significant correlation between the ranks allocated by any given test and that of any of the other tests.

Table 3.2: Wistar rats housed in groups of four were allocated positions in their home cage social hierarchy by three different ranking measures: the amount of sucrose solution consumed in direct competition with cage mates; the time taken to approach a novel food in an habituated open field arena; and the level of social investigation of cage mates when reintroduced after 3h of separation. Data presented here is the rank allocated to each individual animal, within it's home cage group, from each of the three measures.

Table 3.2: Rank position allocated to each animal in the three behavioural tests to determine social status.

Cage	Animal	Competition for Sucrose	Approach to Novel Food	Reintroduction to Cage Mates
A	1	2	1	4
A	2	4	3	2
A	3	3	3	1
A	4	1	3	3
B	1	2	2	2
B	2	1	3	3
B	3	3	1	1
C	1	4	4	3
C	2	1	3	2
C	3	3	2	1
C	4	2	1	4
D	1	1	2	2
D	2	3	2	1
D	3	2	2	3
E	1	4	4	3
E	2	2	1	2
E	3	1	3	4
E	4	3	2	1
F	1	3	3	4
F	2	4	2	1
F	3	1	1	3
F	4	2	4	2

Discussion:

On the first day of ethanol access the individual intake of age-matched controls mirrored that of the experienced drinkers, although further examination of the distribution of intake levels indicates a difference in population spread of ethanol intake. The ethanol naïve animals all consumed more ethanol on the first day of ethanol intake than they did ten days later. However, of particular significance is the fact that the intake on day 1 for the controls was the same as the intake level of the experienced animals, and after 10 days the intake of the control animals had dropped to significantly lower than the experienced animals. The transition from high to low ethanol intake by the control animals over the first 10 days of experience would tend to support the idea of a novelty effect of initial ethanol presentation.

The baseline, group housed, ethanol intake for these animals in discussed in chapter 2, however, the individual intake of these animals showed a high degree of variability between individuals in the same cage and across cages, suggesting perhaps that the group mean used by the original author of the model (Wolffgramm 1990) is hiding a great deal of information about the profile of ethanol consumption of these animals under group housed conditions. A important point to note is the mathematical difference between the method of determination of ethanol intake as a g/kg/24h ratio from group measurements versus individual measurements in a group housed environment. The individual intake measurements are expressed as a mean of the ratio of volume consumed to body weight, while the group housed intake as described by Wolffgramm and Heyne (1995) and used in the long term access part of chapter 2 was calculated as the ratio of the total ethanol consumed to the total body

weight of the animals in each cage. animals in a given home cage. There is a distinct discrepancy between the absolute g/kg/24h value produced by each method.

Social Status

On the first day of introduction to ethanol the age matched control animals showed a significant correlation between the rank allocated by the sucrose competition method and both their individual ethanol intake and preference for the 5% ethanol solution. This correlation indicates that animals ranked highest in the sucrose competition also consumed the most ethanol. Interestingly, however, by the 10^{th} day of ethanol access, this correlation was no longer present and the animals with 82 weeks experience with ethanol did not show a significant correlation between g/kg/24h ethanol intake and the rank allocated by the sucrose competition. There was a significant correlation between consumption of 10% ethanol by the ethanol experienced animals and the rank from the sucrose competition but since the majority of the ethanol consumed was from the 5% ethanol not the 10% ethanol solution (see previous chapter), the relevance of this correlation is not clear.

A surprising part of this correlation, however, is that it indicates that the higher ranked, or dominant, animals had higher ethanol intake than their subordinate cage mates. This is in contrast with the findings of Blanchard *et al.*(1992) and Ellison (1987), both of which showed that subordinate animals drank more ethanol than their dominant counterparts. However, as mentioned earlier, both these studies used sophisticated, naturalistic, environments rather than the highly controlled and standardised cages used here and rankings were based on measures of aggression and wounds received rather than the measure of sucrose competition used here. Also this study uses the more complex ethanol choice of three concentrations plus water

compared to the single ethanol concentration versus water choice of both Blanchard *et al.* (1992) and Ellison (1987). This increased choice complexity and lack of direct competition for ethanol due to the presence of three sources of ethanol may be sufficient to hide any effect of subordinate status on intake.

Taking into account two of the major findings from this study:

a) a correlation between social rank and rank ethanol intake was only seen with ethanol naïve animals when they were first introduced to ethanol, and

b) there was no correlation between ethanol intake and the rank allocated by latency of approach to novel food or investigation upon reintroduction to cage mates,

hese results may actually be more indicative of a relationship along the lines of the findings of Koros *et al.* (1998). These authors reported that the ethanol intake of singly housed (Wistar) rats could be predicted from their saccharin intake: high saccharin intake corresponding to high ethanol intake. Since the present results only show a correlation between ethanol and sucrose consumption on the first day of ethanol intake (when ethanol intake was elevated, possibly due to the novelty of the ethanol solutions), and between sucrose consumption and the 10% ethanol in ethanol experienced animals, then it is possible to conclude that this correlation is actually between the results of two measures of an individual's drive for a rewarding or flavoured solution rather than a correlation between the social status of an individual and its resulting desire for alcohol. On the other side of this issue, however, Goodwin and Amit (1998, 2000) found no correlation in consumption of a saccharin (0.4%) quinine (0.04%) solution and consumption of 10% ethanol when compared within (and between) strains of rats with different ethanol preferences – Fisher, Lewis, Wistar and Kyoto rats.

The lack of correlation between exploratory behaviour and alcohol intake has been shown previously by Blanchard *et al.* (1992) using Long-Evans rats in a visible burrow system. However, the work of Nott and Sibly (1993) using wild-caught female brown rats indicated a strong link between higher exploratory behaviour, decreased time to approach novel foods and dominance. This latter study used the competition between three animals released into a novel arena simultaneously rather than the single animal faced with a novel food in social isolation used here. This, along with the wild-caught nature of their test animals may have made a very significant difference to the findings of Nott and Sibly (1993) as compared with this study and the work of Blanchard *et al.* (1992).

When the three ranking methods used here were compared directly (for the control animals only) there was no correlation between the findings of any ranking method. These results further support the idea that the method of determination of social status within rodent populations is important in the designation of the dominant or subordinate animal (Syme, 1974), and thus is also important in any extrapolation of social status as indicative of other behaviours such as ethanol intake (Hilakivi-Clarke & Lister, 1992), as a model of anxiety related behaviours and neurochemical changes (Watanabe *et al.*, 1995; Blanchard *et al.*, 1991; Spencer *et al.*, 1996), or as a test for anxiolytic efficacy (Joly & Sanger, 1991; Mitchell & Redfern, 1992; Gentsch *et al.*, 1990).

Chapter 4

Psychosocial stress and ethanol preference.

The effect of psychosocial stressors on the ethanol preference ratio of C57 mice.

Introduction

Low preferring mice from the high preferring C57 strain

The C57 strain of mouse, originally bred by C.A. Little in 1921, has been widely used as an alcohol preferring strain (Mclearn and Rodgers, 1959; Phillips and Crabbe, 1991; Belknap *et al.*, 1993). A particular variant of the C57 strain, the C57BL/10 (line ScSn) was originally bred in Bristol University Medical School and from these a colony has now been established at the Drug Dependence Unit at Durham University where the C57BL/10 strain has been bred for over 5 years. Little *et al.* (1999) have demonstrated that any given population of this 'alcohol preferring' strain of mouse actually contains a subpopulation of animals (between 15 and 40% of animals) with a low ethanol preference. Preference for ethanol was defined by these authors as the ratio of fluid consumed from an 8% v/v ethanol solution over the total fluid in a free-choice, two bottle preference method providing 8% v/v ethanol and tap water in two separate bottles 24 hours a day. High preference animals were characterized by an ethanol preference of greater than 0.7, whilst low preference animals had an ethanol preference ratio of 0.4 or lower (Little *et al.*, 1999). A crucial aspect of the definition of these subpopulations within the C57BL/10 strain is the bimodal nature of the population distribution. There are high and low preference animals but very few animals show a 'medium preference' for ethanol, that is a preference ratio between 0.4 and 0.7 (Little *et al.*, 1999).

Further analysis of the biphasic nature of ethanol preference in this strain showed, from a selective breeding programme, that there was no simple genetic link, nor was there any correlation between the basal locomotor activity of individuals and

their ethanol preference. Interestingly the level of locomotion stimulated by intraperitoneal (i.p.) ethanol did not correlate with preference either, which suggests that the difference between the two sub populations was not necessarily based on their sensitivity to the behavioural actions of ethanol. However, daily i.p. injections of saline significantly increased the ethanol preference of the low preferring animals as compared with control animals that were handled but received no injection. This effect was prevented by administration of the CCK_B antagonist CAM 1028 (Little *et al.*, 1999).

Taken together these findings suggest that the C57BL/10 strain may be a very valuable model of the modulation of ethanol intake by stress, and the transition between low ethanol preference (possibly a model of social drinking) and high ethanol preference (a potential model of ethanol intake during addiction). For the following section of my thesis I used animals from the Durham University colony of C57BL/10 mice in two separate studies designed to investigate whether naturalistic social stressors would alter the ethanol preference of low preferring mice, particularly in light of the effects of i.p. saline injections on ethanol preference reported by Little *et al.* (1999).

Stress and ethanol consumption

As discussed in the overall introduction to this thesis, stress is thought to play a major role in precipitating relapse to ethanol drinking and the hormones of the HPA axis (corticosterone and CRF in particular) that are released during stressful events and have been shown to play a crucial role in the modulation of ethanol (Fahlke *et al.*, 1994a, b) and relapse to ethanol drinking (Shaham *et al.*, 1998). As mentioned in the discussion on using the effect of social status on group housed ethanol intake of

Wistar rats (chapter 3), one of the more naturalistic ways to assess the role of stress in the modulation of ethanol consumption is to use stressful social interactions. The work of Little *et al.* (1999) discussed above indicates that low ethanol preferring mice of the C57BL/10 strain may be highly sensitive to stress and so these animals were used to study the effects of social interactions on ethanol intake.

Group housing and ethanol intake

As discussed in Chapter 3 and the overall introduction to this thesis, group housed rodents develop a social hierarchy through aggressive social interaction and the subordinate animal is thought to experience a higher level of stress than the dominant animal. Several studies have demonstrated that the subordinate animal drinks significantly more ethanol than their dominant cage mates (Blanchard *et al.*, 1991, 1992, 1993; Ellison, 1987; Wolffgramm and Heyne, 1995). The first part of this study was designed to investigate whether the ethanol preference of low ethanol preferring C57BL/10 strain is sensitive to the social stress of brief (5 minutes) or extended (1 week) exposure to the aggressive interactions of group housing.

Social defeat in a resident / intruder paradigm

In the second section of this study, social stress was produced by the, inescapable, defeat of the test animal by a resident animal selected to be aggressive in defense of its home cage territory. This particular type of social stress experiment differs from the social status study (Chapter 3) and the previous study in this chapter investigating the effect of group housing on ethanol intake in that aggressive social stress is experimentally produced 'on demand' whereas the methodologies for designating social rank to individuals were designed to elicit classical dominance

behaviours for passive observation and the housing study allows for free fighting on initial introduction to the group housed environment.

The experimental precipitation of social stress through defeat has the advantage over examining the effect of the chronic stress of low social status in that it is easily produced, and readily manipulated in terms of controlling the extent of the defeat (and thus the level of stress) and also the frequency of defeats. By using a standard, unambiguous submissive behaviour as the fixed end point for each defeat encounter, such as the upright submissive posture, so we get an accurate measure of when the attack rate, and corresponding stress level, has reached the threshold to produce this behavioural display. This method should give a more uniform level of stress to each individual than a fixed encounter duration since the major variability in the latter method will be due to the absolute level of attacks and defeats during that period being hugely variable between defeat encounters. In other words, this method uses a measure of the depth of the stress response as a culmination of the subjectively 'filtered' stress stimuli of novelty (as discussed on page 87) and aggression rather than controlling the pre-stress stimuli without knowing the degree of the resulting stress response in each individual.

A single defeat in this paradigm has been shown to induce physiological evidence of stress that include elevation in plasma ACTH and corticosterone levels, increased heart rate and body temperature; fairly short term (1-3h) elevations in glucocorticoids, disrupted circadian rhythmicity in core temperature, blood pressure and heart rate, and decreased access to valuable resources such as food, reproductively active females and preferred habitat (Miczek *et al*, 1991; Tornatzky and Miczek, 1993; Tidey and Miczek, 1996). Korte *et al.* (1995) have also shown that a single defeat attenuated the corticosterone stimulating effect of systemic

administration of the 5-HT_{1A} agonist 8-OH DPAT, whilst Krugers *et al.* (1993) demonstrated an increase in NMDA binding sites and a decrease in AMPA binding sites in the CA3 stratum radiatum following a single social defeat. Repeated defeats over 5 days have also been shown to disrupt the circadian rhythm of core body temperature and heart rate relative to controls (Tornatzky and Mizcek, 1993). Exposure to a resident that had previously defeated the test animal increased the dopamine, DOPAC and HVA levels in the nucleus accumbens (140% of baseline) and prefrontal cortex (160% of baseline) but not lateral striatum of rats (Tidey and Miczek, 1996). Interestingly, exposure to a novel cage or the empty cage of a resident that had previously defeated the test animal also increased the dopamine, DOPAC and HVA levels in the nucleus accumbens and prefrontal cortex but to a lesser degree (130% of baseline) than the presence of the resident animal (Tidey and Miczek, 1996).

Two methodologies were used to precipitate social interactions with differing levels and lengths of aggressive interaction:

1) Temporary group housing (groups of 10) for 5 minutes or 7 days.
2) Single defeat or 5 daily defeats by an aggressive resident animal in a resident intruder paradigm.

The aim of both studies was to investigate whether social defeat could significantly increase the ethanol preference of low preferring C57BL/10 mice.

Common Methods for both experiments

Animals used:

The animals used in these studies were male C57BL/10 mice bred in Durham University weighing between 25 and 30g at the start of the experiment. All animals were singly housed in standard laboratory cages under normal phase lighting conditions (lights on 0700 hours and off at 1900 hours), room temperature was 20°C and humidity 40 - 60%. Animals were provided with *ad libitum* access to standard laboratory chow and tap water at all times. 148 mice were screened for ethanol preference and 84 of the low preferring animals were used (40 for the group housing experiment and 44 for the resident intruder experiment). In order to aid identification of individual animals, two days prior to any exposure to ethanol, all animals were 'tagged' with a subcutaneous microchip (Avid technologies) carrying a unique identification number that could be read by passing a hand held 'reader' over the back of each animal. This was necessary to ensure that each animal was returned to their own starting home cage and not to a cage with the territorial scents of another animal in it following the social stress encounters (group housing or social defeat).

Ethanol preference measurements and screen for low preference test animals

For the measurements of ethanol preference animals were given access to two bottles one containing 8% v/v ethanol the other containing tap water. Fluid intake was measured every Monday, Wednesday and Friday and the amount drunk from each bottle was used to calculate the ratio of 8% ethanol consumed to the total fluid consumed (the ethanol preference ratio). Measurements were made for three weeks to screen for the individuals with low ethanol preference. The mean ethanol preference ratio for the third week of ethanol access was used to designate the individual animals

as low (preference ratio of less than 0.4), high (preference ratio 0.7 and above) or intermediate (preference ratio greater than 0.4 up to 0.7) drinkers. The screening produced a total of 98 low preference animals, 35 high preference animals, and 15 intermediate preference animals.

1) The effect of temporary group housing on the ethanol preference of singly housed, low ethanol preferring, C57BL/10 mice.

Aim:

The aim of this study was to investigate whether brief (5 minute) or extended (1 week) exposure to group housing would alter the ethanol preference of singly housed, low ethanol preferring, C57BL/10 mice.

Methods:

Changes in housing condition

Mice were singly housed for the three weeks of preference screening and 40 of the low preferring animals were split into 4 test groups each with 10 members. The last week of preference screening was designated as the 'baseline' week. On Monday of the first week following preference screening (test week 1) the housing conditions were manipulated in the following manner:

"Control" group: (n=10)

Singly housed mice were left with continuous access to ethanol (8% v/v) and tap water in their home cage for the entire experimental period (four weeks).

"5 min" group: (n=10)

On the Monday of test week 1, all the mice in this test group were placed together in one cage for 5 minutes to allow sufficient time for introductory aggression but not the establishment of any social structure, and then returned to their original home cages for the rest of the experimental period (four weeks).

" Novelty" group: (n=10)

On the Monday of test week 1, singly housed mice were individually placed in novel cages for 5 minutes and then returned to their original home cage for the rest of the experimental period (four weeks).

"Group housed" group: (n=10)

On the Monday of test week 1, all the mice in this test group were placed together in one cage for 1 week. On the Monday of test week 2 (one week later) the mice were returned to their original home cages for the rest of the experimental period (three weeks).

Housing changes were made at 1000 hours, during the light phase for all groups, and ethanol intake was measured continuously using the two bottle preference method as before for test conditions 1, 2 and 3 (control, 5min and novelty groups). Individual ethanol intake was measured Monday, Wednesday and Friday for 3 weeks (test weeks 2-4) following test week 1 (the week when housing conditions were manipulated). Standard laboratory chow was available *ad libitum* to all groups at all times. During the week long period of group housing, the "group housed" group was provided with 2 bottles containing 8% v/v ethanol and 2 bottles of tap water in order to allow all individuals continued free access to ethanol and water. Ethanol intake during this period of group housing has not been included in the analysis of this experiment owing to the difference in sampling methods of group housed intake measurements when compared to singly housed measurements for the same animals (chapter 3). Following the return to individual housing, the ethanol preference

measurements were resumed for this group also and ethanol intake from the periods of single housing before (baseline week) and after (weeks 2-4) the week of group housing (test week 1) has been used to investigate the effect of the group housing period on ethanol intake of individually housed mice.

Statistical Analysis:

Mean ethanol preference for each test week was compared between groups for each week using a one-way analysis of variance. The mean ethanol preference for each week was compared across the 5 weeks of the experiment, within each housing / test group, with a one-way analysis of variance. *Post hoc* analysis was carried out using a Student Newman Keuls test in both analyses. Analysis of the absolute g/kg/24h ethanol intake was compared using an analysis of covariance because the absolute baseline ethanol intake was significantly different between groups. Thus comparisons between groups and across time were made using the baseline intake as a covariate.

Due to the variability of the data produced, *post hoc* power analysis was performed using GPower (copyright: Faul and Erdfelder, 1992) to determine the n value required in each group to produce an 80% chance of a significant result at the $P < 0.005$ level.

Results:

The effect of changes in housing condition on the ethanol preference of low preference C57 mice

After the three week preference screening period, the mean individual preference ratio ± sem was 0.24 ± 0.03 ("control" group), 0.24 ± 0.02 ("5 min" group), 0.26 ± 0.03 ("novelty" group), 0.26 ± 0.02 ("group housed" group). There was no significant difference between the ethanol preference (figure 4.1) of the "control" group and any of the test groups, or between any of the test groups, on any of the test weeks. There was no significant change in the ethanol preference of individual groups across the experimental period (i.e. within groups analysis by time).

Comparison of the g/kg/24h ethanol consumption, however, showed a significantly lower ethanol intake by the control group (1.9 ± 0.3 g/kg/24h) compared with the "5 min" (3.5 ± 0.7 g/kg/24h) and "novelty" (3.7 ± 1.1 g/kg/24h) groups on the baseline week ($P < 0.05$; figure 4.2). The ethanol intake of these groups were not significantly different at any other time point. The "group housed" group also had a significantly lower g/kg/24h ethanol intake (1.3 ± 0.2 g/kg/24h) compared to the "5 min" and "novelty" groups on the baseline week. The ethanol intake of these groups was not significantly different at any other time point when this baseline difference was used as a covariate in the analysis. The "control" group and the "group housed" group showed no significant difference in their ethanol intake at any time point.

In a comparison within test groups over the duration of the experiment (i.e. within groups analysis by time), the mean g/kg/24h ethanol consumption was significantly elevated over the baseline week in both the control group and the "group housed" groups on test weeks 3 and 4 ($P<0.05$) (figure 4.2). In the same comparison,

there was no significant difference over the duration of the experiment in the ethanol intake of either the "novelty" or the "5 min" groups. However, the total fluid intake was significantly elevated on test week 4 as compared to the baseline week, and test weeks 1 and 2 ($P<0.05$) for both the "5 min" and "novelty" groups (figure 4.3). The "control" and "group housed" groups showed no significant change in their total fluid consumption over time. Power analysis of these data indicated that a n value of 780 per test group would be required for there to be a significant difference between the ethanol consumption of these test groups, suggesting that, despite the apparent difference in the graphical representation of the data, the difference is not verging on significance and a small increase in the group size tested would not have increased the likelihood of finding a significant result.

Figure 4.1: Low ethanol preference C57 mice were singly housed and given access to 8% v/v ethanol and water. After three weeks preference screening, the last week of which is designated as the baseline week here, animals were either left singly housed (◆), n = 10; exposed to a clean cage for 5 minutes (■), n = 10; group housed for 5 minutes (▲), n=10; or group housed for one week (week 1) (×), n = 10. Ethanol preference was measured three times a week and is presented as the ethanol preference ratio : g of ethanol consumed / g total fluid consumed.

Figure 4.1: The effect of changes in housing condition on the ethanol preference of low preferring C57 mice.

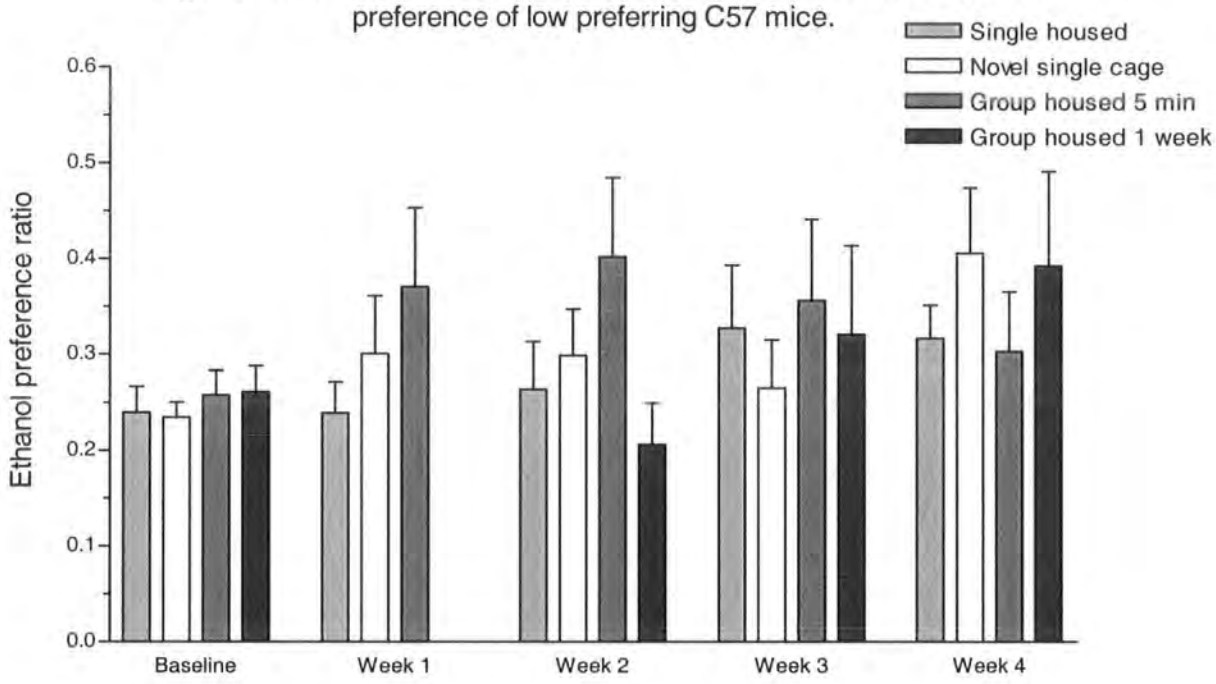


Figure 4.2: Low ethanol preference C57 mice were singly housed and given access to 8% v/v ethanol and water. After three weeks preference screening, the last week of which is designated as the baseline week here, animals were either left singly housed (◆), n = 10; exposed to a clean cage for 5 minutes (■), n = 10; group housed for 5 minutes (▲), n=10; or group housed for one week (week 1) (×), n = 10. Ethanol intake was measured three times a week and is presented as ethanol intake : g of ethanol consumed / kg body weight / 24h.

* = $P<0.05$ for the ethanol intake of "control" and "group housed" groups compared with that of the "novelty" and "5min" groups.

† = $P<0.05$ for the ethanol intake of the "control" and "group housed" groups on week 3 compared with intake on the baseline week.

Figure 4.2: The effect of changes in housing condition on the ethanol intake of C57 mice.

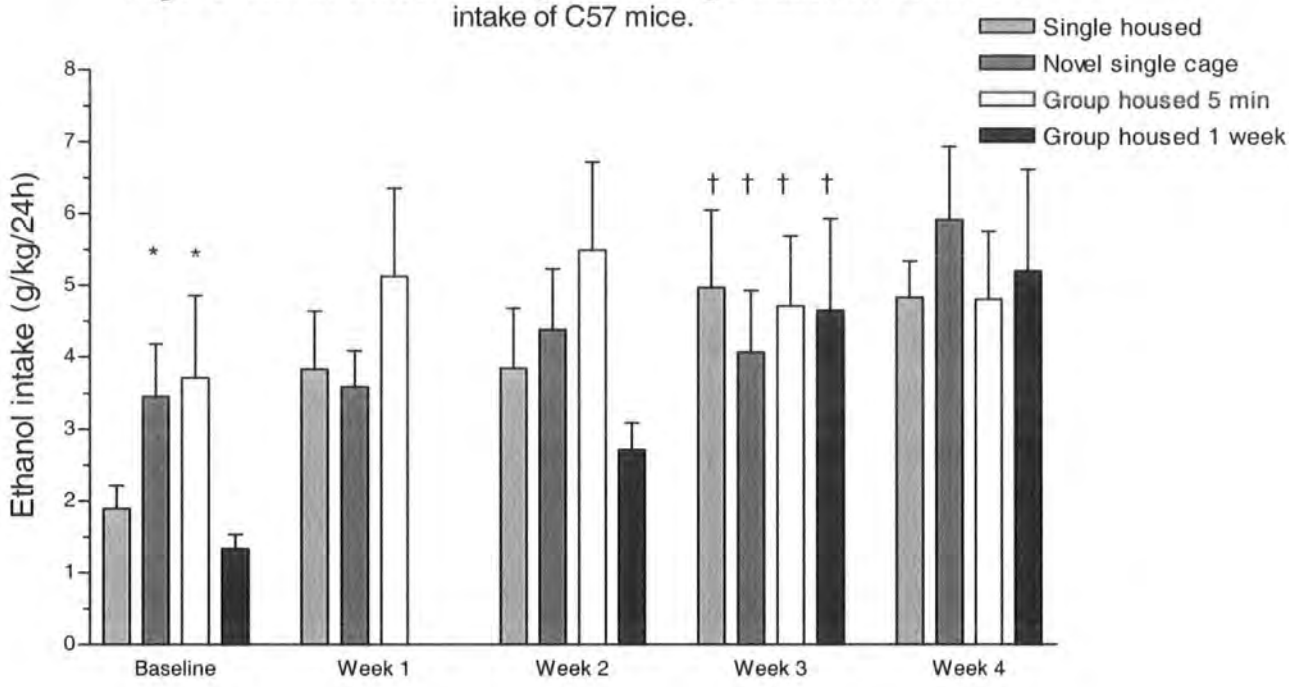
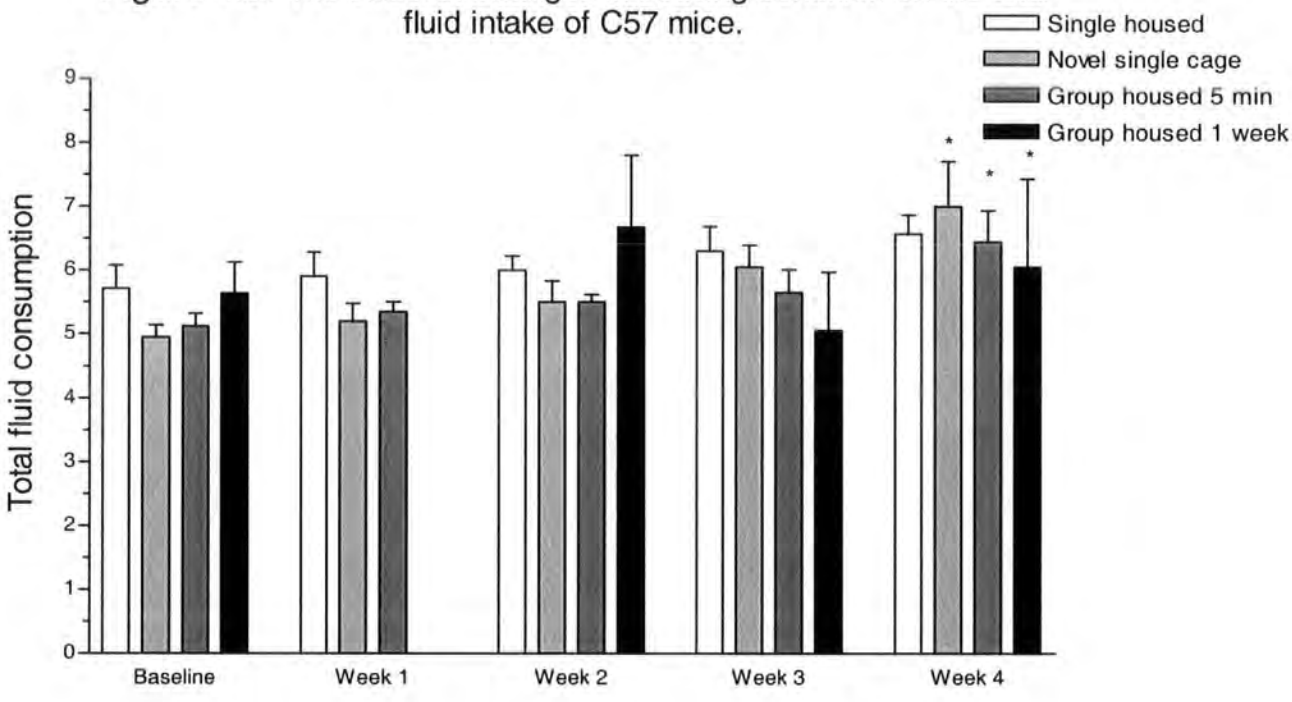


Figure 4.3: Low ethanol preference C57 mice were singly housed and given access to 8% v/v ethanol and water. After three weeks preference screening, the last week of which is designated as the baseline week here, animals were either left singly housed (◆), n = 10; exposed to a clean cage for 5 minutes (■), n = 10; group housed for 5 minutes (▲), n=10; or group housed for one week (week 1) (✕), n = 10. Fluid intake was measured three times a week and is presented here as g total fluid consumed / day.

* = $P<0.05$ for the total fluid intake of the "novelty" and "5min" groups on week 4 compared with that on the baseline week and test weeks 1 and 2.

Figure 4.3: The effect of changes in housing condition on the total fluid intake of C57 mice.



2) The effect of social defeat on the ethanol preference of singly housed, low ethanol preferring, C57BL/10 mice.

Manipulations of housing conditions (group versus singly housed) had no effect on ethanol preference. However, the aggressive interaction may not have been sufficient to produce a large stress response and elevation in corticosterone levels. Thus a more controlled stressor was tested in a high aggression situation – the resident intruder paradigm.

Aim:

The aim of this study was to investigate whether single or repeated (daily for 5 days) defeats in a resident intruder paradigm would alter the ethanol intake of singly housed, low ethanol preferring C57BL/10 mice.

Methods:

Resident Intruder Encounters

The resident intruder encounters were based on the established methods of Rodgers and Randall (1986). The basic principle revolves around the aggressively territorial behaviour of the resident animal in its home cage when confronted by an unknown intruder and the removal of any escape route to the intruder animal.

Resident animals were screened to select for animals that would attack quickly and effectively whenever an unknown mouse was placed in their cage. This was done by daily encounters with smaller mice as intruders in the resident's home cage and the speedy removal of the intruder as soon as the upright submissive posture was displayed. The resident group selected for this experiment were selected against TO

mice because these white mice were available and easily distinguished from the black C57 residents (the strain of mouse defeated during selection of the resident animals is not crucial to the selection regime). The upright submissive posture is characterised by an upright body position similar to a rear except that the forelimbs are held rigid towards the attacker. The defeated animal will crane around to face the attacker (who is usually biting the intruders back) without actually moving their hind limbs and will stare very wide eyed at the attacker. The main overriding characteristic of this defeat posture is that the animal remains very rigid in both body and limbs. In order to prevent escape of the intruder animal during resident training and test encounters, an artificial wall was added around the singly housed mouse home cage used because the animals being attacked could (and would) jump out of the standard cage. After three daily encounters over consecutive days, resident animals that did not defeat the intruder within 6 minutes were removed from the selection procedure because they were not aggressive enough to use as resident animals. In this study, 15 mice were screened and 11 were suitably aggressive to remain in the pool of resident animals. The optimum defeat time was within 1 minute from introduction of the intruder, and all resident animals achieved this at least once during the screening procedure.

The aggressive encounters between low ethanol preferring intruder animals and aggressive residents were carried out under red light conditions as follows. All animals were moved in their racks to the test room antechamber and left to settle for 1 hour. Resident animals were randomly labeled from 1 to 11 so that no intruder met the same resident animal twice in the repeated defeat group. The established home cage for the resident animal was placed in the test room under a low light video camera and the intruder to be defeated brought into the room. The intruder was placed directly into the home cage of the resident animal in the opposite corner to that

of the resident. The entire encounter was recorded using a fast frame speed so that the tape could be slowed down at a later date for subsequent analysis of the number and frequency of bites by the resident. The time taken for the resident to attack the intruder and elicit an upright defeat posture was measured and the intruder removed as soon as this posture was displayed in order to prevent any injury being caused by the resident animal. There was no time limit to the test encounter since the end point for the encounter was aggressive defeat resulting in a display of the upright submissive posture.

Control animals underwent exactly the same procedure except that they were placed in a clean empty cage to control for any effect on ethanol preference resulting simply from the change of cage as opposed to changes in ethanol preference induced by introduction to the established, scent marked, home cage of an aggressive resident and the subsequent defeat of the intruder. Full defeat and display of the upright submissive posture took between 10 s and 6 minutes. In order to control for this variation in the length of exposure to a new cage environment, control animals were paired with defeated animals and exposed to the novel cage for precisely as long as the paired defeated animal was exposed to the residents cage.

The final test groups used were as follows:

1) A single defeat on day one of the 'defeat week' – n=11
2) A single exposure to a novel cage on day one of the 'defeat week' – n=11
3) Five daily defeats by different resident animals on Monday to Friday of the 'defeat week'- n=11

4) Five daily exposures to a novel cage on Monday to Friday of the 'defeat week' – n=11

The last week of the preference screen was designated as the 'baseline' week and the week that the social encounters took place (the first week after the baseline week) was designated the 'defeat' week. Preference measurements continued for a further 4 weeks after the defeat week and these weeks were designated weeks 1-4.

Statistical Analysis:

Ethanol preference and g/kg/24h intake measurements were compared within groups across time using a one-way analysis of variance. One-way analysis was also used to compare the ethanol intake and preference of all four test groups on each test day. *Post hoc* analysis was carried out using a Student Newman Keuls test.

Results:

The effect of single and repeated defeat on ethanol intake

During the defeat encounters, all intruders were defeated within 6 minutes. The mean time taken from the start of the encounter to an unambiguous display of the upright submissive posture was 2 min 47 s ± 6s. A single defeat or a single exposure to a novel cage resulted in a gradual increase in ethanol intake (figure 4.4) and preference (figure 4.5) over the defeat week and the subsequent 2 weeks. This increase, however, was not significantly greater than the baseline week until the 2nd week after the 'defeat week' and then only in the group exposed to the novel cage ($P<0.05$). The animals that were either defeated once daily for 5 days or exposed to a novel cage daily for 5 days showed a similar increase in ethanol intake (figure 4.6) and preference (figure 4.7), reaching significance (over baseline intake) during the

'defeat week' for both the defeat and novel cage control groups ($P<0.05$), and remaining significantly high until a peak during the 2nd week following the 'defeat week' ($P<0.05$ for weeks 1 and 2 following defeat). By the 3rd week following the 'defeat week', the repeated novel cage controls and both the single defeat and single novel cage controls had returned to an ethanol intake and preference level that was not significantly different to baseline levels. However, the animals that had been exposed to 5 daily defeats maintained a significantly elevated ethanol intake for the last 2 weeks of intake measurements ($P< 0.05$ compared to the baseline week). The mean intake for this group on the last experimental week (week 4 after the 'defeat week') was 4.57 g/kg/day ± 1.09 as compared to 2.69 g/kg/day ± 0.61 for the repeated novel cage controls. The total fluid intake for all 4 groups declined gradually over the 6 weeks of the test period (figures 4.8 and 4.9), however, the preference ratio of ethanol intake to total fluid (and absolute ethanol intake) was the same on the baseline week and weeks 3 and 4 for all groups except the group that had experienced repeated defeats, suggesting that this decrease in total fluid was not a significant factor in the effects of defeat and novelty on ethanol intake and preference.

Figure 4.4: Low ethanol preference C57 mice had access to ethanol (8% v/v) and water for three weeks (third week = baseline). Individually housed animals were exposed to either: a single social defeat (◆), n = 10, or a clean novel cage (■), n = 10. Ethanol intake was measured weekly during the defeat period (Defeat) and for a subsequent 4 weeks (Week 1 – 4). Data is presented as g of ethanol consumed / kg body weight / 24h.

* = $P<0.05$ for the ethanol intake of the single novelty group on week 2 compared with the baseline intake.

Figure 4.5: Low ethanol preference C57 mice had access to ethanol (8% v/v) and water for three weeks (third week = baseline). Individually housed animals were exposed to either : a single social defeat (◆), n = 10, or a clean novel cage (■), n = 10. Ethanol intake was measured weekly during the defeat period (Defeat) and for a subsequent 4 weeks (Week 1 – 4). Data is presented as the ethanol preference ratio: g of ethanol consumed / g total fluid consumed.

* = $P<0.05$ for the preference ratio of the single novelty group on week 2 compared with the baseline week.

Figure 4.4: The effect of single defeat on the ethanol intake of low preferring C57 mice.

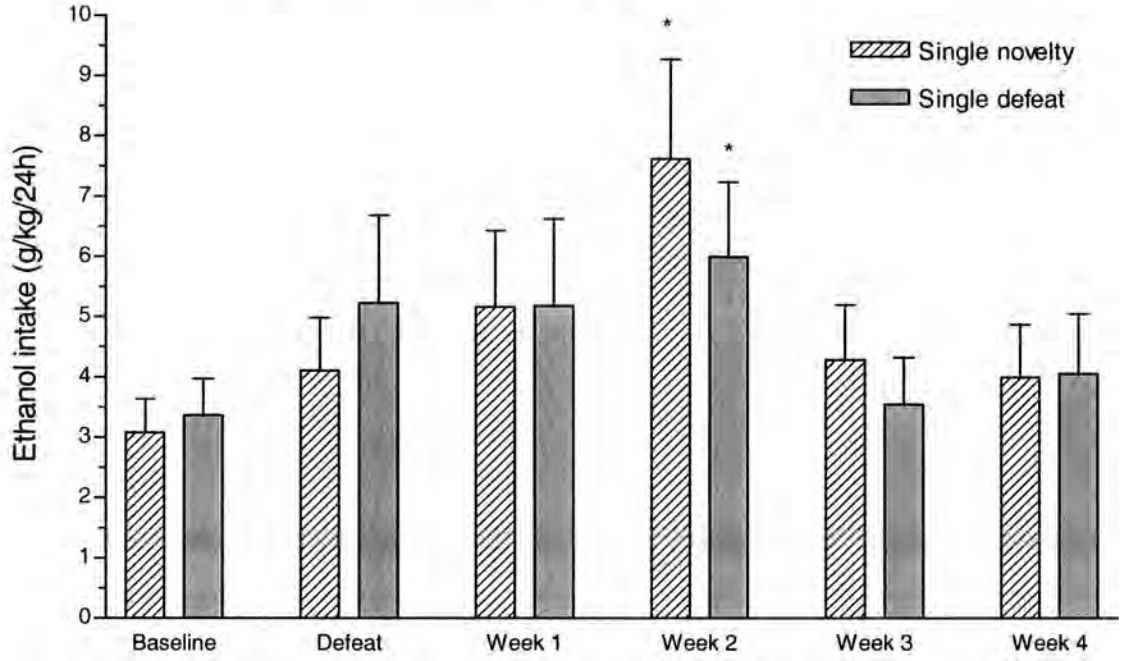


Figure 4.5: The effect of single defeat on the ethanol preference ratio of low preferring C57 mice.

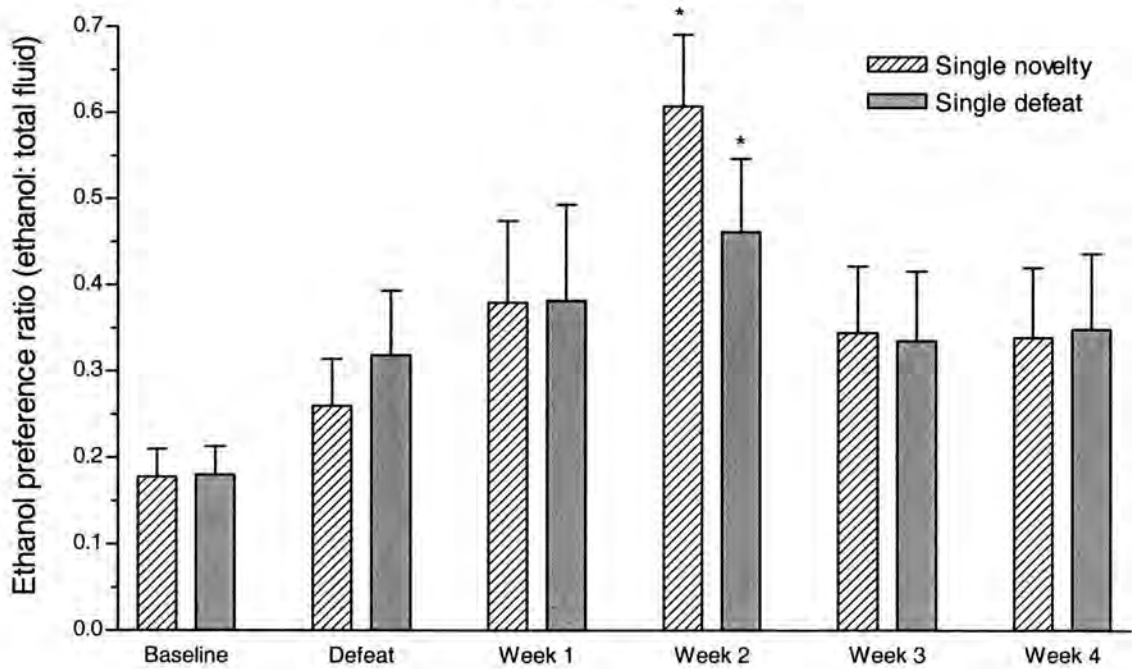


Figure 4.6: Low ethanol preference C57 mice had access to ethanol (8% v/v) and water for three weeks (third week = baseline). Individually housed animals were exposed to: five daily social defeats (△), n = 10, or a clean novel cage daily for five days (×), n = 10. Ethanol intake was measured weekly during the defeat period (Defeat) and for a subsequent 4 weeks (Week 1 – 4). Data is presented as g of ethanol consumed / kg body weight / 24h.

* = $P<0.05$ for ethanol intake for the repeated defeat and repeated novelty groups when compared to their own baseline intake.

† = $P<0.05$ for the repeated defeat group only compared to their baseline intake.

Figure 4.7: Low ethanol preference C57 mice had access to ethanol (8% v/v) and water for three weeks (third week = baseline). Individually housed animals were exposed to: five daily social defeats (▲), n = 10, or a clean novel cage daily for five days (×), n = 10. Ethanol intake was measured weekly during the defeat period (Defeat) and for a subsequent 4 weeks (Week 1 – 4). Data is presented as ethanol preference ratio: g of ethanol consumed / g total fluid consumed.

* = $P<0.05$ for ethanol intake for the repeated defeat and repeated novelty groups when compared to their own baseline intake.

† = $P<0.05$ for the repeated defeat group only compared to their baseline intake.

Figure 4.6: The effect of 5 daily social defeats on the ethanol intake of low preferring C57 mice.

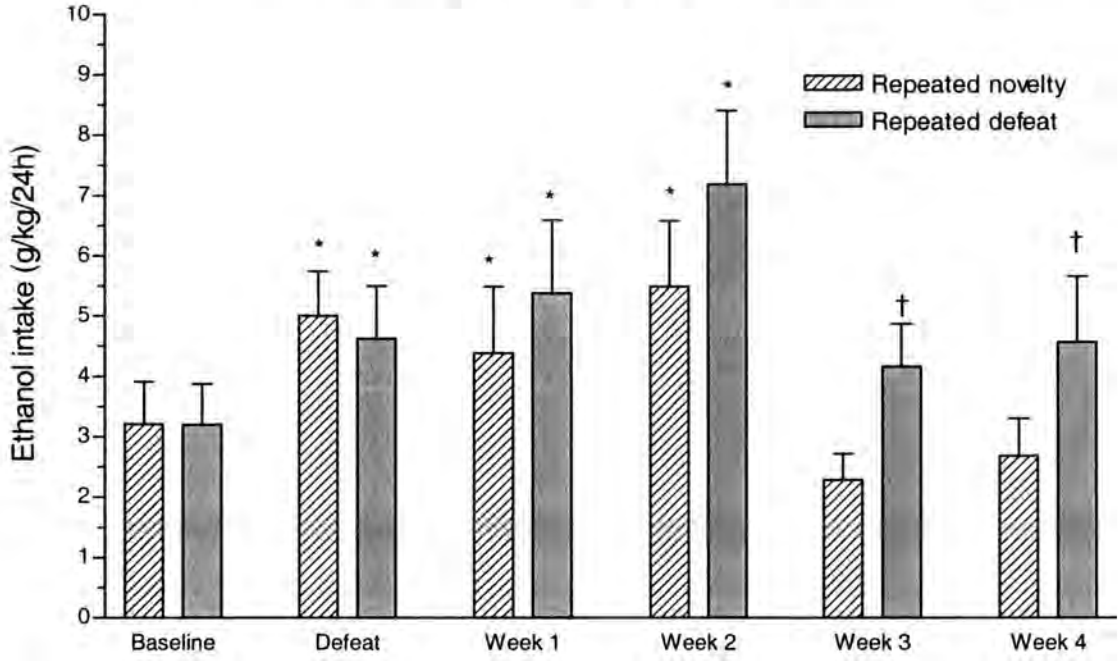


Figure 4.7: The effect of 5 daily social defeats on the ethanol preference ratio of low preferring C57 mice.

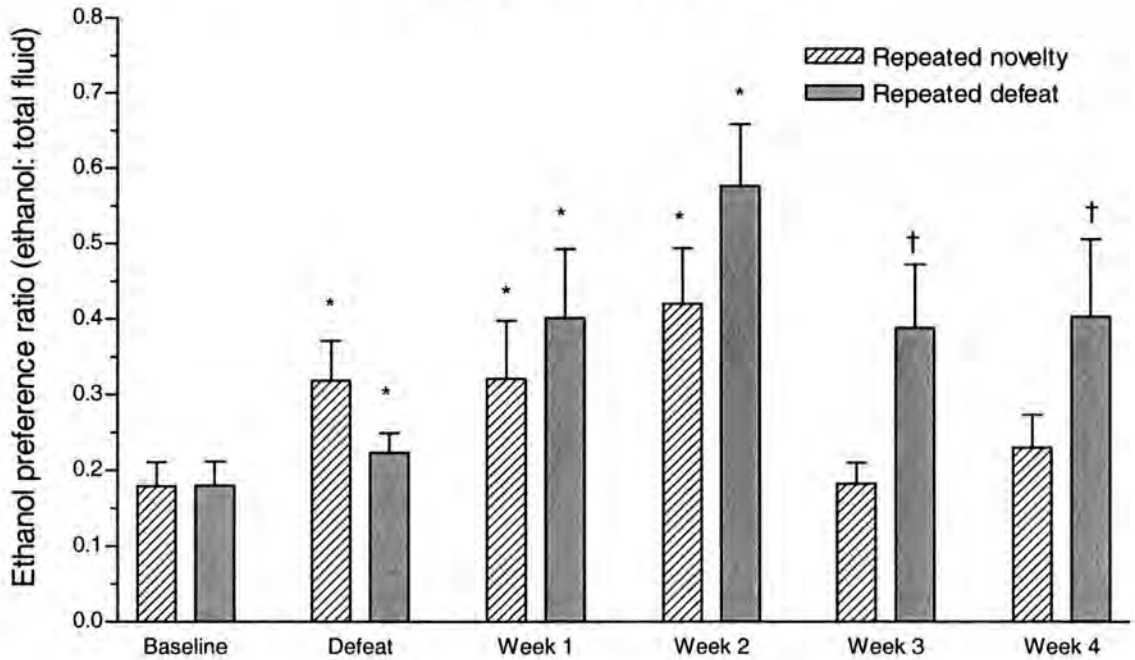


Figure 4.8: Low ethanol preference C57 mice had access to ethanol (8% v/v) and water for three weeks (third week = baseline). Individually housed animals were exposed to either: a single social defeat (◆), n = 10, or a clean novel cage (■), n = 10. Fluid intake was measured weekly during the defeat period (Defeat) and for a subsequent 4 weeks (Week 1 – 4). Data is presented as g of total fluid consumed.

Figure 4.9: Low ethanol preference C57 mice had access to ethanol (8% v/v) and water for three weeks (third week = baseline). Individually housed animals were exposed to: five daily social defeats (▲), n = 10, or a clean novel cage daily for five days (×), n = 10. Fluid intake was measured weekly during the defeat period (Defeat) and for a subsequent 4 weeks (Week 1 – 4). Data is presented as g of total fluid consumed.

Figure 4.8: The effect of single defeat on the total fluid intake of low preferring C57 mice.

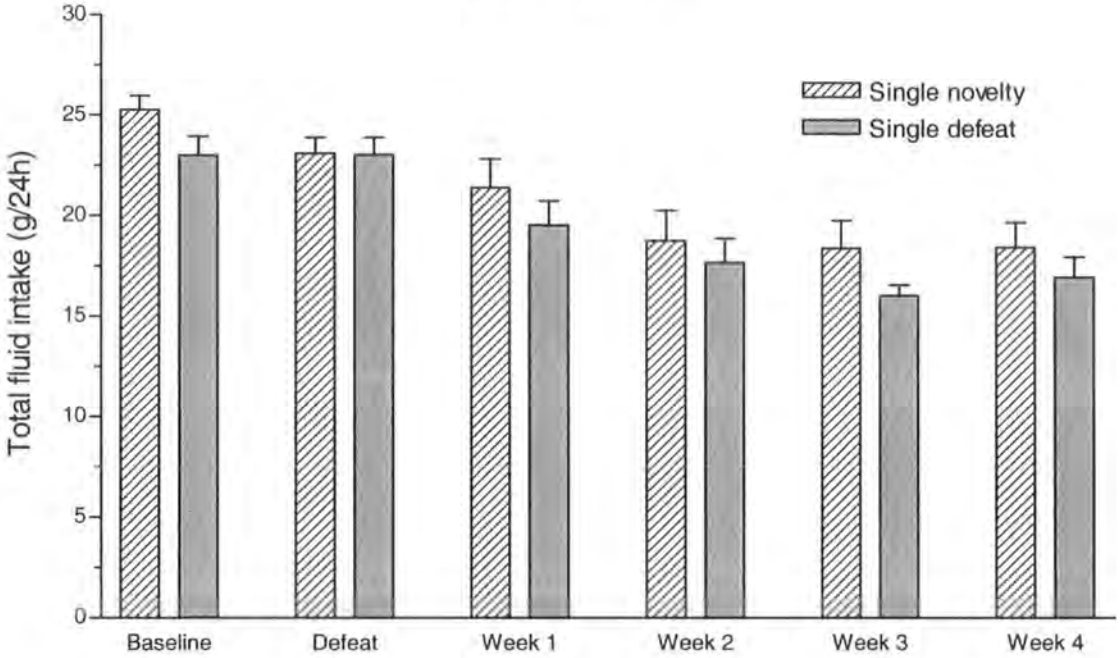
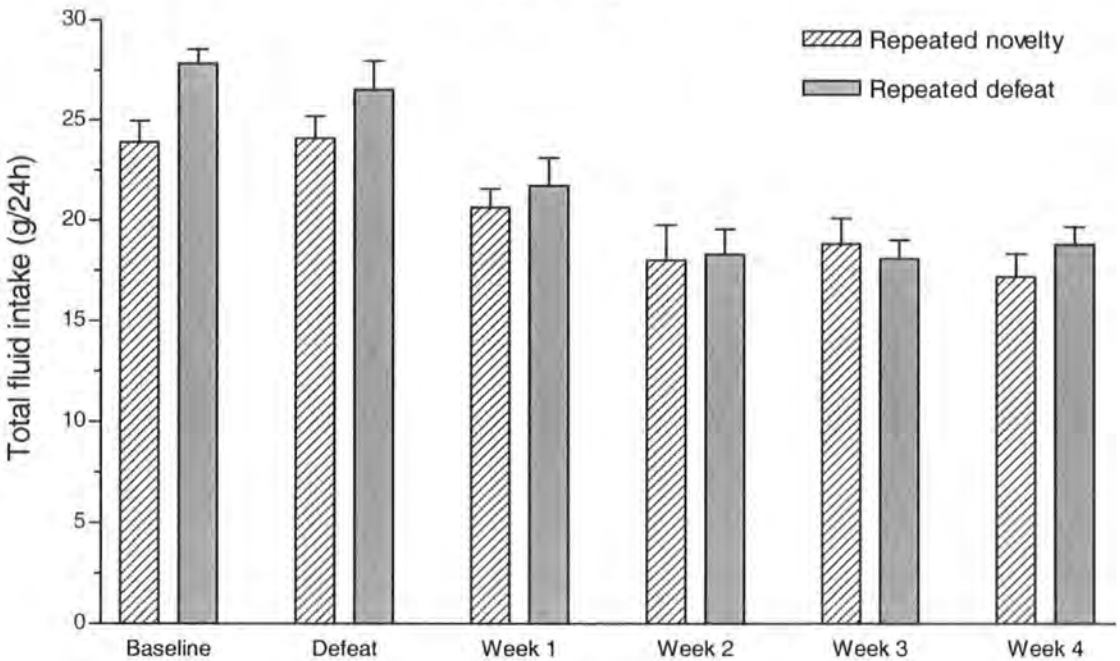


Figure 4.9: The effect of 5 daily social defeats on the total fluid intake of low preferring C57 mice.



Discussion:

The effect of temporary group housing on the ethanol preference of singly housed, low ethanol preferring, C57BL/10 mice.

This experiment was designed to investigate whether changes in housing conditions would affect the ethanol preference ratio of the low ethanol preference sub-population of the C57BL/10 strain of mice. However, there was no significant effect of the manipulations of housing conditions on ethanol preference ratio for any of the groups tested. Further analysis of the data did, however, show that the absolute ethanol intake (g/kg/24h) of the animals that were group housed for 1 week and the control group, that was not manipulated at all, was significantly increased relative to the baseline week by test weeks 3 and 4. This increase in intake of the control animals would tend to negate any possible relevance of the increase in ethanol intake following 1 week of group housing (figure 4.2).

The original aim of the experiment was to investigate the effect of housing on ethanol preference of low preference mice, and the primary conclusion is that there was no effect of ethanol preference. However, the difference in the g/kg/24h ethanol intake on the baseline week undermines this conclusion since the test groups were consuming different amounts of ethanol and so may have reacted to their housing conditions differently. The results from this study underline the necessity for parallel analysis of ethanol preference ratio, absolute ethanol intake and total fluid in order to produce test groups that are truly comparable, despite the fact that logically one would expect that, if the ethanol preference is not different and the total fluid consumption is not different then the ethanol intake should not be different between groups. The only explanation I can propose is that, while these three measures are inextricably linked, they are still mean values and so the variation of each group may produce two measures (e.g. ethanol preference and total fluid intake that are not significantly

different between groups) yet the variation in the third measure (e.g. ethanol intake) may be just sufficient to produce significant differences in the test groups.

The C57BL/10 strain of mice have been shown to be sensitive to the physical stress of repeated i.p. injections of saline (Little *et al.*, 1999), resulting in elevated ethanol preference. However, the results from my study show that the introduction to group housing for a week (or 5 minutes) did not produce a similar effect on ethanol preference, suggesting perhaps that the social stress of aggressive interactions in a group housed environment was not equivalent to repeated saline injections. Whether these two forms of stressor differ in the degree of stress experienced by the individual animals or whether the social stress of group housing involves slightly different hormonal and neuronal systems to those activated by the physical stress of repeated i.p. injections is not clear.

The effect of social defeat on the ethanol preference of singly housed, low ethanol preferring, C57BL/10 mice.

Ethanol intake was elevated over the first three weeks following exposure to a single novel cage, a single defeat, repeated exposure to novel cages or repeated social defeat. However,in all groups except the group exposed to 5 daily defeats, this increased intake returned to baseline levels by the 4th post-test week. The ethanol intake and preference of animals defeated 5 times did not return to baseline levels during the 6 weeks of measurements, suggesting that repeated social defeat (but not single social defeat) produced a longer term change in the modulation of ethanol intake than exposure to the novel environment did.

When investigating the effect of the social defeat experience on dopamine release and metabolism, Tidey and Miczek (1996) demonstrated that exposure of control animals to a novel cage or a scent marked but empty cage increased dopamine, DOPAC and HVA levels in the nucleus accumbens and prefrontal cortex

to 130% of baseline levels, whilst the presence of a resident animal that had previously defeated the test animal resulted in greater increases in dopamine, DOPAC and HVA (140% of baseline in the nucleus accumbens, 160% of baseline in the prefrontal cortex). This elevation of dopamine release in novel or scent marked cages correlated well with exploratory behaviour, however, the increased release in the presence of an aggressive resident coincided with a decrease in locomotion and the increase is more likely linked to an increase in attention and arousal than motor stimulation (Tidey and Miczek, 1996).

Given the link between the mesolimbic dopamine system and drug abuse onset and relapse, the rapid stimulation of dopamine release in this system by stressful environmental stimuli supports a link between such environmental stimuli contributing to the initiation of relapse to drug abuse such as ethanol intake. The results presented in this thesis also support this idea, since repeated social defeat resulted in the maintenance of an elevated ethanol consumption precipitated by a novel environment. However, the effects of social defeat reported by Tidey and Miczek (1996) are over shorter time periods (hours) compared to the longer time course (weeks) of the preference results presented here. Indeed, the elevated ethanol preference seen in these results does not reach significant levels until well after the cessation of measurements by Tidey and Miczek (1996) in their study.

Chapter 5

Chronic ethanol intake and neuronal function.

Electrophysiology of the Ventral Tegmental Area following chronic ethanol intake

Introduction:

Midbrain dopamine neurons project to many different regions (page 13). However, only certain pathways are involved in the modulation of reward and self-administration of drugs of dependence and the particular dopamine pathways of interest in the study of ethanol reward and dependence seems to be the mesolimbic and mesocortical pathways (see chapter 1 for more details).

The majority of dopamine cells in these medial forebrain bundle projections originate in the ventral tegmental area (VTA), and ethanol has been clearly demonstrated to have effect here on both cell firing rate (Brodie *et al.*, 1990, 1999) and dopamine release (terminal and somatodendritic; Wozniak *et al.*, 1991 and Robertson *et al.*, 1991 respectively). There also appears to be a significant role for 'stress' in the modulation of ethanol's effects on the dopaminergic system. Social stress and artificial stressors such as foot-shock and restraint stress have been shown to elevate ethanol intake, whilst Yavich and Tiihonen (2000) have demonstrated a clear link between repeated social defeat and ethanol's potentiation of dopamine release in the nucleus accumbens.

Using a Wistar rat midbrain slice, Cho and Little (1999) demonstrated that corticosterone (100nM to 1μM) potentiated the increases in firing rates of the VTA dopamine cells induced by 5 and 15μM NMDA in a calcium dependent manner (50nM corticosterone had no effect). Corticosterone had a biphasic effect on AMPA- (10μM) and kainate- (30μM) induced increases in VTA firing rate, however. 50nM

corticosterone had no effect, 100nM corticosterone decreased the AMPA and kainate stimulated firing rate, while 500nM and 1μM both increased the AMPA and kainate stimulation of VTA firing. 2μM corticosterone, however, increased only AMPA stimulated VTA firing and decreased kainate stimulated VTA firing (Cho and Little, 1999). These effects of corticosterone were blocked by the glucocorticoid antagonist, RU 38486, but the mineralocorticoid agonist, aldosterone, had no effect on NMDA-induced increases in firing rate, indicating that corticosterone modulates the effects of excitatory amino acids on VTA firing rate via type II corticosteroid receptors (Cho and Little, 1999).

Greater than 90% of the neuronal population in the striatum and nucleus accumbens contain glucocorticoid receptor immunoreactivity (Zoli *et al.*, 1990) and Overton *et al.* (1996) used *in vivo* recordings of the electrical activity of the VTA to demonstrate an effect of corticosterone (equivalent to 13.4μg/100 ml plasma, ~0.4μM) on glutamate-induced changes in burst firing activity of dopaminergic cells. Their results showed that adrenalectomy significantly reduced the basal firing rate of the dopaminergic cells of the VTA, and corticosterone replacement reversed this effect (Overton *et al.*, 1996). These results are similar to the effects of adrenalectomy and corticosterone replacement on ethanol consumption (Fahlke *et al*, 1994a, b, 1995) but disagree with the lack of effect of corticosterone on basal firing reported by Cho and Little (1999). However, this latter study applied corticosterone to slices from adrenally-intact animals and thus any genomic effects of the basal corticosterone before slice preparation would have still been present, and it is possible that the intracellular, high affinity mineralocorticoid receptors may still be occupied by the endogeneous corticosterone.

Manley and Little (1997) reported that 24 hours after withdrawal from chronic ethanol containing liquid diet, the stimulation of locomotor activity by amphetamine or cocaine was not significantly different to that of animals that had received control diet (ethanol naïve animals). After ten daily amphetamine or cocaine injections, animals that had received ethanol diet showed a significantly higher level of locomotor stimulation with both amphetamine and cocaine than animals that had received control diet (Manley and Little, 1997). When amphetamine and cocaine administration was started 6 days after withdrawal from ethanol diet, amphetamine, but not cocaine, produced a significantly greater level of locomotor activity in ethanol withdrawn animals than the ethanol naïve controls. After 10 days of repeated amphetamine or cocaine administration, both cocaine and amphetamine produced a significantly higher level of locomotion in the ethanol withdrawn animals compared with ethanol naïve controls (Manley and Little, 1997). Two months (60 days) after withdrawal from ethanol liquid diet, amphetamine and cocaine produced a higher level of locomotion in ethanol withdrawn animals than controls, although the increased locomotion was statistically significant only in the case of cocaine and not with amphetamine. After 10 days of repeated amphetamine or cocaine administration, both drugs produced a significantly higher locomotion in ethanol withdrawn animals than controls (Manley and Little, 1997).

Administration of nicotine following withdrawal from two months of ethanol containing liquid diet showed no effect of ethanol diet on nicotine-induced locomotor stimulation (Watson and Little,1999). However, after 28 days of nicotine administration, animals that had received ethanol liquid diet showed a significantly elevated nicotine-induced locomotor stimulation compared to animals that had received control diet (Watson and Little, 1999). These results indicate that chronic

ethanol treatment has long lasting effects on the dopamine systems modulating locomotor activity long after cessation of ethanol access (over 60 days) and beyond any signs of the physical withdrawal syndrome.

Bailey *et al.*(2000) demonstrated that following chronic ethanol containing liquid diet, the spontaneous activity of the dopaminergic cells of the VTA (when recorded in an *in vitro* slice environment) had decreased to the point that only one cell could be detected 24h after withdrawal from ethanol. However, application of NMDA at a concentration that had no effect alone in ethanol naïve slices (5μM) stimulated the ventral tegmental area sufficiently to produce detectable cell firing 24 h after withdrawal from chronic ethanol. Six days after withdrawal from ethanol, the basal firing of the VTA neurons was detectable but still depressed, however by 2 months after ethanol withdrawal the VTA firing rate had returned to ethanol naïve control levels (Bailey *et al.*, 1998). At these time points, following administration of ethanol as part of a liquid diet, changes in monoamine turnover have been reported (Bailey *et al.*, 2000). These results indicate that there are long lasting neurological changes in the dopamine system following chronic ethanol containing liquid diet in addition to the behavioural effects observed by Manley and Little (1997). Interestingly, however, the results of Bailey *et al.* (2000) show changes in neuronal activity lasting up to and including 6 days after withdrawal from ethanol diet, but not present at the 2 month (60 day) time point, whereas the sensitisation to locomotor stimulation by amphetamine and cocaine observed by Manley and Little (1997) lasted through the 2 month time point, suggesting that these two changes are probably not causally linked 2 months after withdrawal from ethanol diet.

Relapse to ethanol consumption can be stimulated by stressful events (page 6) implying changes in the sensitivity to HPA hormones and / or abstinence from

ethanol. If this were the case, then the VTA may show altered sensitivity to corticosterone after withdrawal from ethanol as in the model used by Bailey *et al.* (2000) and Manley and Little (1997).

Aims:

The overall aim of this study was to investigate whether, 6 days after withdrawal from chronic ethanol containing liquid diet, there was any effect of chronic ethanol treatment on corticosterone-induced potentiation of elevations in VTA firing rate produced by NMDA.

In addition to the main aim of this study, a preliminary investigation was carried out into the relevance of the phase of the light dark cycle during which the midbrain slice was prepared on the sensitivity of the VTA neurons to NMDA. Circulating corticosterone levels fluctuate in a diurnal rhythm (Doe *et al.*, 1954; Scheving and Pauly, 1966) and it was important to know whether the time of slice preparation would be relevant to the effect of NMDA either alone or in conjunction with corticosterone.

Methods:

Animals

Male TO mice were used for the electrophysiological study in line with the previous electrophysiological work of Bailey *et al.*, (1998, 2000). All animals were group housed (6-10 per cage) in standard laboratory cages at 20°C, 40 - 60% humidity on a 12h/12h light dark cycle (normal phase lights on 0700 off 1900h; reverse phase lights on 1900 off 0700 h) and weighed between 30 and 35g.

Artificial Cerebrospinal Fluid (Electrophysiology buffer).

Brain slices were kept in artificial cerebrospinal fluid (aCSF) throughout the duration of the experiment. The aCSF used was carbonate buffered and provided a pH 7.4 when bubbled heavily (i.e. saturated) with 95% oxygen, 5% carbon dioxide). Daily requirements of aCSF were made fresh each morning from 10x concentrated stock solutions. However, in order to prevent the precipitation of calcium carbonate and other insoluble salts from the concentrated stock solutions, these were stored as 3 different solutions which were added in equal parts to distilled water to provide the aCSF:

Chemical	Final Concentration
NaCl	124 mM
KCl	3.25 mM
KH_2PO_4	1.25 mM
D-glucose	10 mM
$MgSO_4$	2 mM
$NaHCO_3$	20mM
$CaCl_2$	2mM

Brain Slice Preparation for Electrophysiology

Brain slices were produced each day from 2 animals to provide parallel control slices for all drug conditions. Animals were 'lightly' anaesthetised using halothane and then decapitated. Owing to the nature of the spontaneously active neurons of the VTA it was necessary to keep the level of anaesthesia as light as possible and to decapitate the animals and remove the brain at the first sign of loss of righting reflex. Deeper anaesthesia seemed to result in the loss of baseline firing in this brain region (these findings have been informally confirmed by members of the Bonci laboratory at the University of California, San Francisco who have been making whole cell patch clamp recordings from VTA neurons).

Following decapitation, the scalp and dorsal surface of the skull were swiftly removed and the brain was doused with ice-cold artificial cerebro-spinal fluid (aCSF). Using a small spatula with rounded ends, the cranial nerves were severed with the brain *in situ* and then the brain was lifted out of the skull and placed in ice-cold, oxygenated aCSF. Oxygenation was accomplished through light bubbling with 95% oxygen, 5% carbon dioxide. In the mouse preparation (in contrast to the rat preparation also used in the laboratory) the meninges were left intact as they were delicate enough not to cause problems with slicing, yet damage would have been caused during their removal. The whole brain was placed in a petri dish mounted on ice and containing oxygenated aCSF for manual production of a slicing block. The brain was placed on its dorsal surface with the spinal cord region pointing towards the researcher and, using a single edged razor blade, the brain was trimmed to a suitable block for slicing in the following manner:

1) A coronal / vertical cut was made through the base of the spinal cord, removing the cerebellum and most of the hindbrain (figure 5.1a)
2) A coronal cut 10° from vertical was made through the optic chiasm (inclination of cut was 10° away from researcher cutting downwards and towards the brain stem) (figure 5.1a)
3) The block was placed on the posterior surface produced by the first cut and a vertical cut was made through the dorsal 3rd ventricle and the dorsal portion was discarded (5.1b)
4) The block was laid on the dorsal cut surface and the cortex teased / cut off both the sides of the block.

This dissection resulted in a block which was mounted on its posterior face, with the uncut ventral surface facing outward, using superglue. The uppermost face (the anterior cut face) looked similar to figure 5.2a and following the removal of several redundant slices exposed the VTA as in figure 5.2b.

The block was mounted under ice-cold, gassed, aCSF in a vibratome (Intracell) for slicing. Coronal slices were cut at 350μm thickness with a slow blade progression rate (approximately 2mm / minute) in a ventral to dorsal direction. Slices were floated off the blade using fine artists brushes and lifted using a spatula with smoothed edges to prevent tearing of the slice. Only one useable slice was produced from each mouse brain due to the small size of the VTA and the thickness of the slice. Each slice was transferred directly into the tissue holding chamber containing oxygenated aCSF at 35°C (described below) and left to rest and equilibriate to 35°C for 60 minutes.

Figure 5.1a: The first two cuts made to produce the midbrain block for slicing.

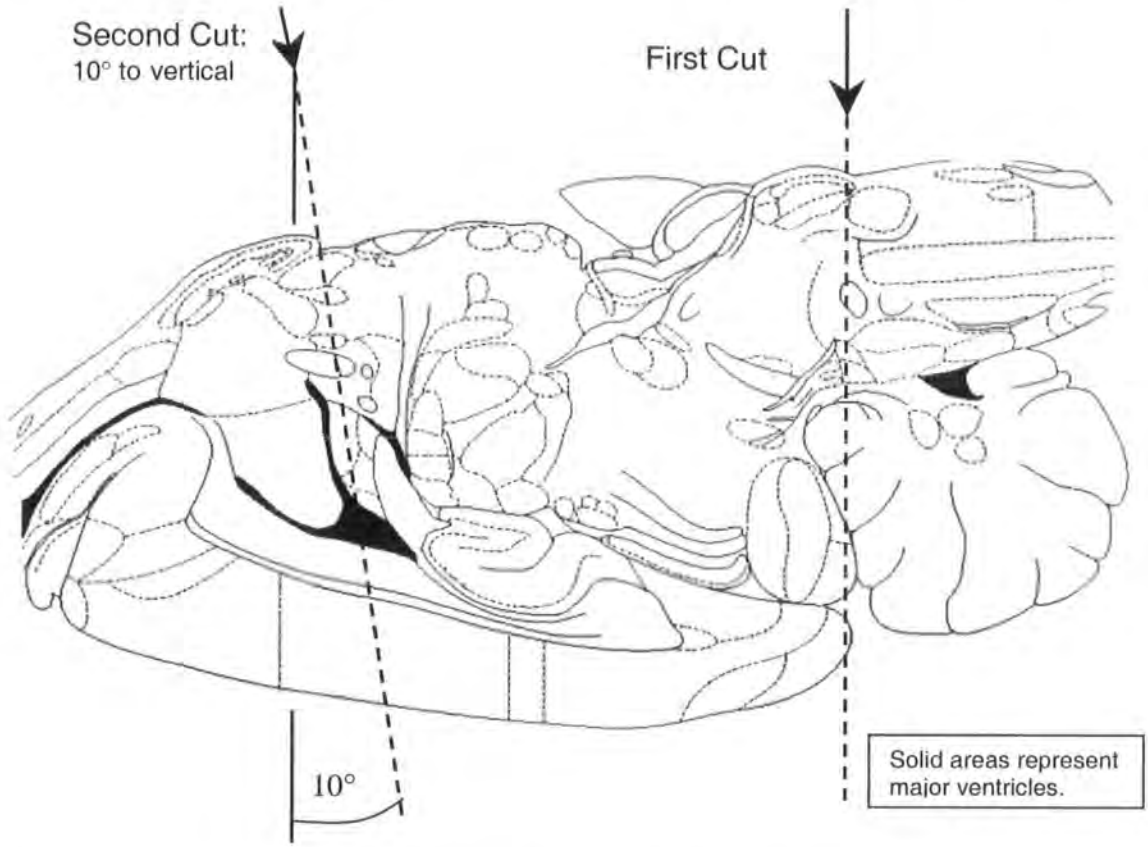


Figure 5.1b: The third cut to produce the midbrain block for slicing

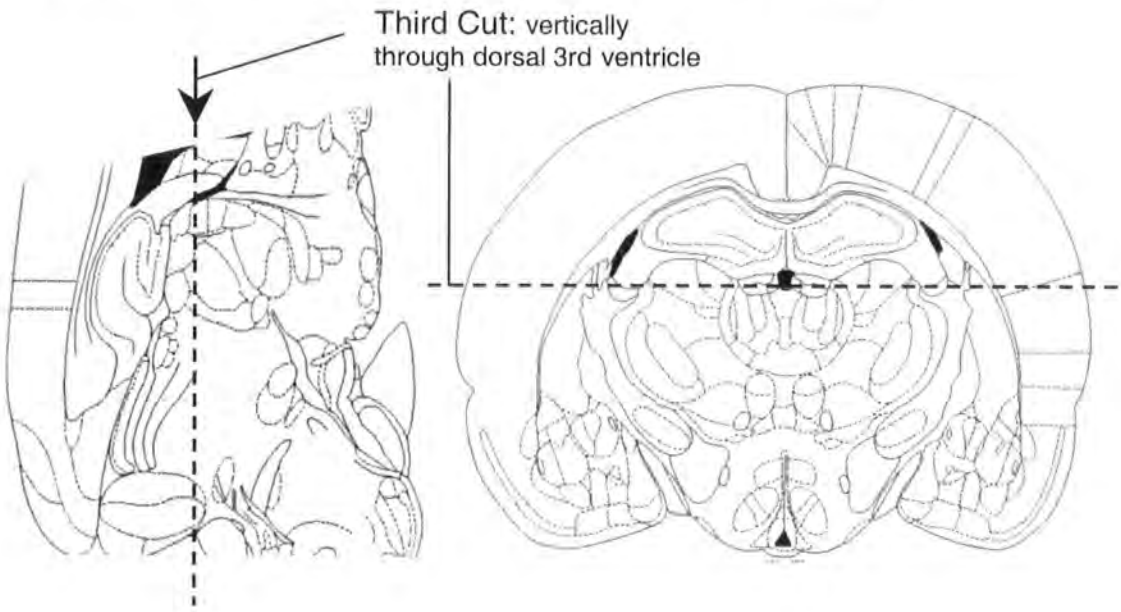


Figure 5.2a: Top view of the midbrain block prior to slicing.
Regions labeled are purely for orientation and are not crucial to slice.

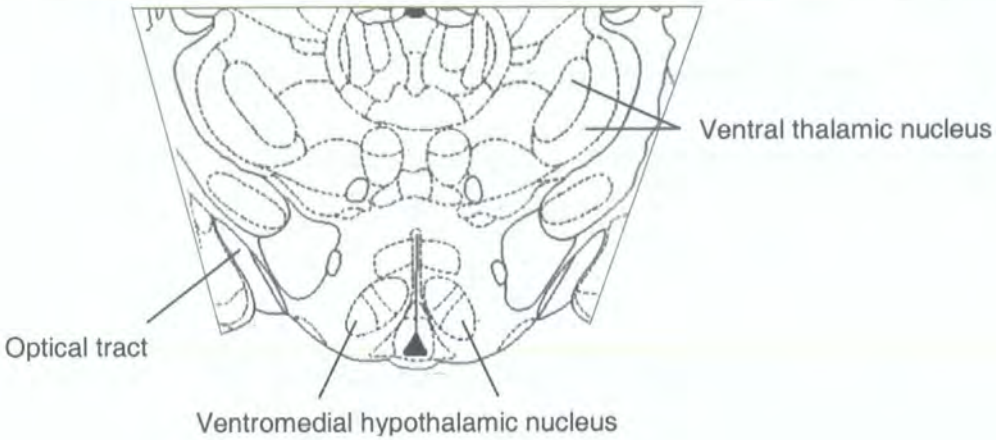
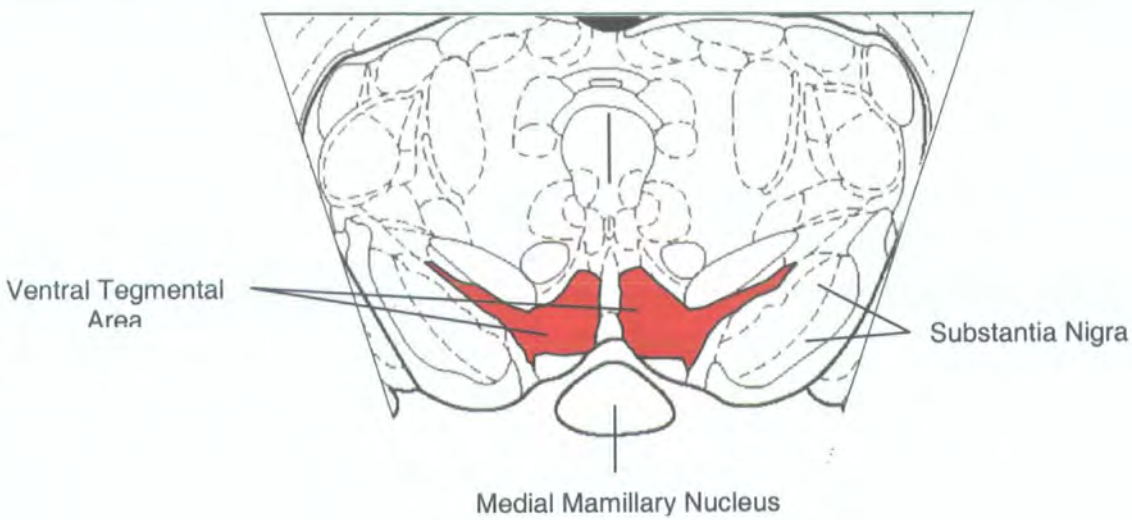


Figure 5.2b : Surface view of the midbrain slice containing the Ventral Tegmental Area used for electrophysiological recordings.



Tissue Chamber for Electrophysiological Recordings

The tissue chamber used for the electrophysiological recordings was a continuous perfusion, interface chamber with warm humidified air-flow over the slice surface, and consisted of a heating chamber underneath a tissue bath (figure 5.3a). The heating chamber contained distilled water heated to 35°C with a closed loop from an external water bath. All aCSF and drug solution tubing rose through this chamber to pre-warm these solutions. The tissue bath was a small hollow in the lid of the heating chamber with aCSF and drug delivery tubing rising vertically up through the base and a wick based drainage system at the outlet (figure 5.3b).

Slices were placed in the tissue bath on a tissue holder which was a rectangular perspex frame with a coarse mesh grid stretched over it (women's tights). In order to provide a clean interface between the slice and the tissue holder and to maintain even aCSF coverage, the slice was placed on a small piece of lens tissue and then placed on the mesh. The aCSF level was maintained at such a height that the surface of the slice remained wet but was not submerged in aCSF. This was achieved through manipulation of the height of the wick at the outlet end of the tissue bath. The most efficient and controllable wick was made from heavy gauge filter paper (Whatman B).

The rate of aCSF delivery was critical for the survival of the spontaneously active neurons (most probably due to the provision of oxygen dissolved in the aCSF) and a flow rate of 2.5 ml/min was optimal. Large (2 L) volumes of aCSF were stored outside the Faraday cage in conical flasks in a water bath at 35°C and were constantly gassed with 95% oxygen, 5% carbon dioxide. The pre-warmed, pre-gassed aCSF was delivered to the tissue bath via a peristaltic pump (situated outside the Faraday cage). In order to limit the pulsatile flow of the aCSF coming from this pump, a multi-

barreled peristaltic pump was used (resulting in more, but smaller pulses) and the aCSF flow was broken by delivery into a small (1ml) conical flask. Artificial CSF entered the flask above the level of aCSF in the flask and then the tip of the exit tube was placed in the aCSF just above the base of the flask. As each pulse of aCSF was pumped into the flask the air in the flask absorbed some of the pulse pressure, whilst the outlet tube received a far more continuous flow of aCSF.

Delivery of aCSF and drug solutions to the tissue bath was achieved with vertically rising steel tubing that passed through the lid of the heating chamber and the base of the tissue bath. These tubes (two per tissue bath) projected up high enough out of the base of the tissue bath that the aCSF outlet was always above the level of the tissue bath fluids. This meant that aCSF was delivered as a low pressure cascade back down the outside of the steel tube, rather than as a turbulent flow directly into the buffer (as would be produced with a submerged delivery system).

In order to keep the exposed surface of the slice moist and oxygenated, the heating chamber was bubbled with 95% oxygen, 5% carbon dioxide and small openings in the lid of the heating bath allowed the humidified, oxygen rich air to rise up. A series of baffles and glass slides then directed and contained this humidified air over the tissue bath and slice. While very effective, this system required careful manipulation of the gassing rate of the heating chamber since over-gassing resulted in an increase in evaporation resulting in excess condensation in the tissue bath area. This condensation of distilled water in the vicinity of the slice was detrimental to slice survival and maintenance of spontaneous activity.

Figure 5.3a: Tissue chamber side view including heating chamber and tissue bath.

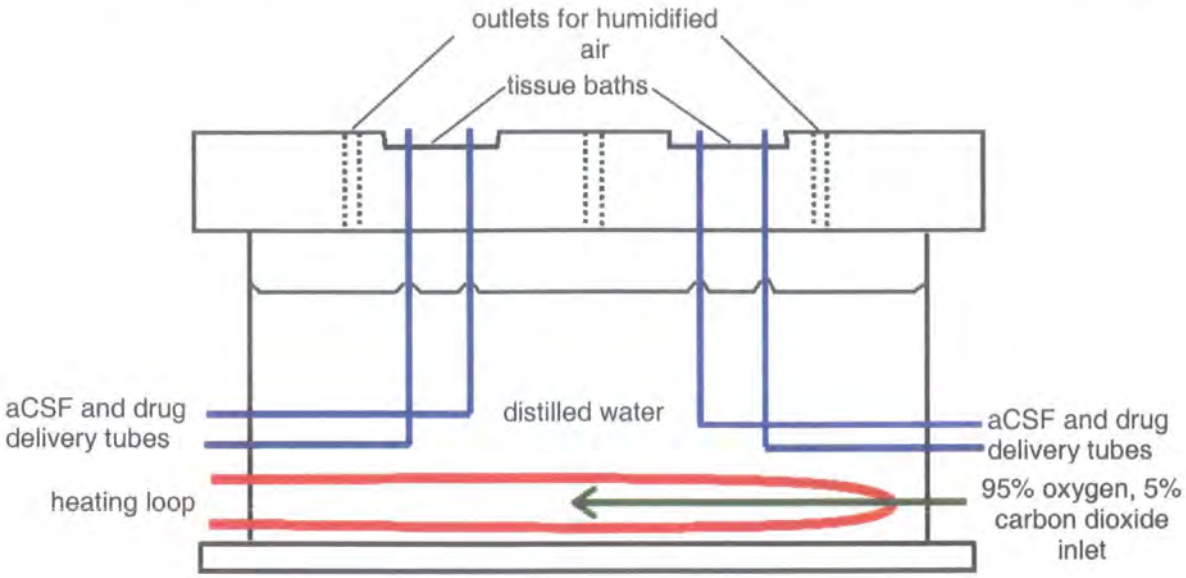
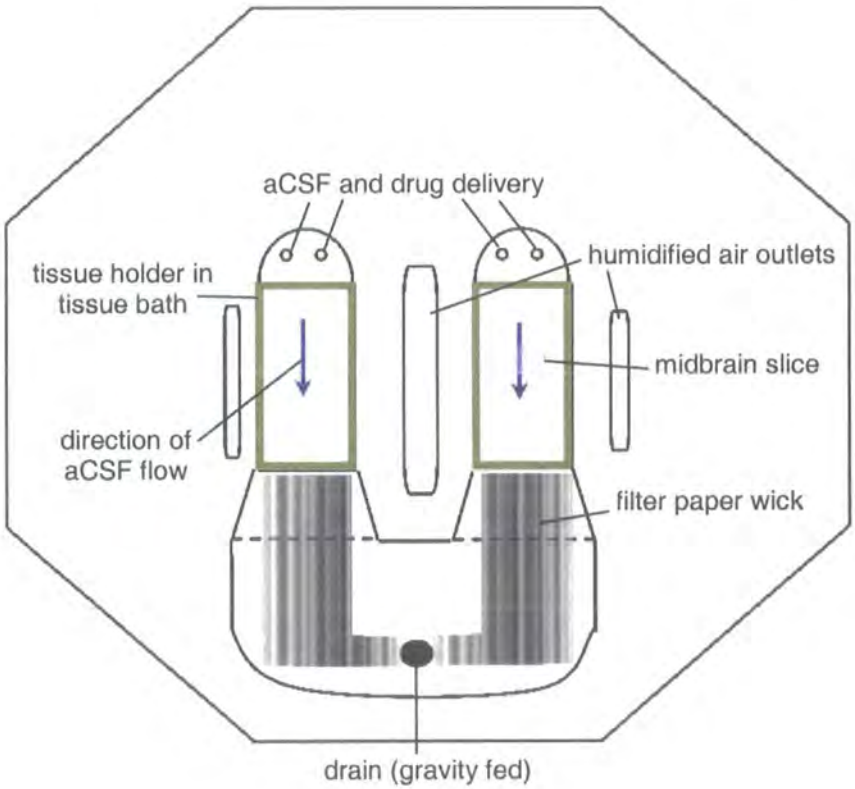


Figure 5.3b: Plan view of tissue chamber showing tissue baths.



Electrophysiological Recording

Extracellular single unit recordings were made of the spontaneously active cells of the VTA using an Axoclamp 2B amplifier (Axon Instruments) and HS2A unity-gain headstage (Axon Instruments). Borosilicate electrodes were used with a tip resistance between 7 and 14 MΩ produced by a single pull programme on a horizontal, humidity controlled, Flaming/Brown electrode puller (P87 – Sutter Instruments). Electrodes were filled with 3M NaCl and the headstage junction was produced using a chlorided silver wire. The bath was grounded via the headstage ground using a chlorided silver wire placed in the tissue holder. The analogue data output from the Axoclamp was converted to a digital signal by MacLab/8 data acquisition hardware (ADInstruments) connected to a PPC Apple Macintosh computer for real time graphical display and data storage using the MacLab/8 software (ADInstruments). Gross microelectrode positioning was performed using a standard coarse micromanipulator, whilst accurate penetration of the slice was achieved using a remote stepper, making steps of 2μm coaxial to the plane of the electrode.

The electrode tip was descended into the slice until the characteristic action potential shape of the spontaneously active, dopamine sensitive, cells was seen (Figure 5.4a). In addition to the action potential shape, the rate of baseline firing was used to confirm neuronal type. Preliminary studies indicated that cells identified in this manner were sensitive to dopamine. All the cells inhibited by 20μM dopamine had a spontaneous activity of between 0.7 and 5 Hz. However, for later experiments dopamine was not applied to cells since 10 and 20μM dopamine inhibited firing and completely stopped cell firing in some (but not all cells). Also the washout period was 25 –35 minutes before full basal firing returned. This extensive pre-test for

dopamine sensitivity absorbed much of the experimental time available with any one cell and so was omitted from later experiments. When the action potential height was greater than the noise (usually 4 – 10 fold greater) and the firing was regular and reliable (action potential height was not drifting too much) then the cell firing was recorded for 1 minute (figure 5.4b). Once a cell had been found the slice was left to settle with the electrode in place for 20 minutes. This rest period also confirmed that the cell was going to continue firing regularly and that the slice and electrode tip positions were stable relative to each other. Following the resting period, drugs were administered in the perfusion medium (aCSF) by switching between delivery tubes and replacing the bathing solution. Whilst this did not produce very fast changes in the drug levels in the bath (typically taking about 4 minutes for maximal drug effects, figure 5.5), the bathing concentration was known and fixed (as compared to local application of drug onto the slice) and the level of effect of each concentration used was comparable across cells, implying reliable penetration of the slice by the drugs examined here.

Methodological difficulties and time constraints

The method of slice preparation and extracellular recording described above is well established in our laboratory and several researchers have used it successfully prior to my PhD studies. However, during the course of my investigations, the reliability of the method deteriorated significantly. I finally narrowed the source of the problem down to faulty plumbing of the water still which had resulted in accumulation of salt residues in the still and contamination of the water used to prepare the aCSF. Upon discovery of this problem an alternate water source was immediately found and, with the new water supply, the reliability of extracellular slice recordings was recovered. However, the development and diagnosis of this problem

was a fairly long process and as a result I had a very limited amount of time left to complete the electrophysiological studies as I had planned them. As a result, some of the group sizes in the following studies (particularily those following liquid diet) are too small for valid statistical comparison, however, all data has been included as a significant proportion of my PhD time was spent planning and carrying out these studies.

The effect of time of slice preparation (reverse or normal phase) on NMDA activation of VTA cell firing.

Slices were prepared from TO mice that had been housed on either reverse phase (12h:12h cycle, lights on at 7pm) or normal phase (12h:12h cycle, lights on 7am). This allowed for a comparison between the effect of NMDA on slices from animals during their awake and asleep phases respectively. Slices were prepared as described above and extracellular recordings made from dopaminergic neurons. The effect of 5, 20 ,50 and 100μM NMDA was investigated by exposing the slice to aCSF containing the respective concentration of NMDA for 6 minutes and then washing out with pure aCSF for 15 minutes (sufficiently long enough for a return to the baseline firing rate). For both reverse phase and normal phase animals n=8. Doses were applied in a cumulative manner (i.e. increasing concentration) to avoid the effect of desensitization caused by a higher dose interacting with a subsequent low dose of NMDA. The dose response to NMDA was also repeated in the presence of corticosterone with reverse phase animals (n=5).

Owing to the large file sizes produced by continuous recordings of the entire procedure, only the last 1 minute of the baseline period prior to drug administration and the last 1 minute of the drug application period was analysed. This should also

have allowed sufficient time for complete drug penetration into the slice irrespective of any differences in how deep within the slice each cell recorded from was situated.

The effect of ethanol on VTA cell firing

Slices were prepared from TO mice that had been housed on reverse phase (12h:12h cycle, lights on at 7pm). Slices were prepared as described above and extracellular recordings made from dopaminergic neurons. The effect of 40, 80, 160 and 320mM ethanol was investigated by exposing the slice to aCSF containing the respective concentration of ethanol for 6 minutes and then washing out with pure aCSF for 15 minutes (sufficiently long enough for a return to the baseline firing rate).

The effect of chronic ethanol intake on NMDA and ethanol induced stimulation of VTA firing rate

Six days after withdrawal from chronic ethanol intake as a liquid diet (procedure described below), the effect of NMDA and ethanol on the firing rate of VTA neurons was examined. Slices were prepared as described and extracellular recordings made from dopaminergic cells. Concentrations of ethanol and NMDA were chosen from the concentration response curves presented here. A corticosterone concentration was chosen based on the work of Cho and Little (1999) that indicated the effectiveness of corticosterone at 100nM to 1μM. Owing to the length of experimental procedure and large number of controls necessary, only one concentration could be examined and 200nM was chosen as an effective concentration from the work of Cho and Little (1999). Once a spontaneously active cell had been found, slices were exposed to repeated NMDA concentrations

(20,50uM) +/- ethanol (40, 80mM) +/- corticosterone (200nM) in one of the following procedures:

Control: NMDA only: n = 8 for control diet n = 8 for ethanol diet	Ethanol & NMDA: n = 3 for control diet n = 4 for ethanol diet	Corticosterone & NMDA: n = 6 for control diet n = 7 for ethanol diet	Corticosterone, ethanol and NMDA: n = 4 for control diet n = 6 for ethanol diet
1. 20 min rest after finding cell;	1. 20 min rest after finding cell;	1. 20 min rest after finding cell;	1. 20 min rest after finding cell;
2. 15 min aCSF;	2. 15 min aCSF;	2. 15 min 200nM corticosterone *	2. 15 min 200nM corticosterone*
3. 20μM NMDA for 6 min;	3. 20μM NMDA for 6 min;	3. 20μM NMDA for 6 min;	3. 20μM NMDA for 6 min;
4. 50μM NMDA for 6 min;	4. 50μM NMDA for 6 min;	4. 50μM NMDA for 6 min;	4. 50μM NMDA for 6 min;
5. aCSF wash 15 min;	5. aCSF wash 15 min;	5. corticosterone / aCSF wash 15 min;	5. corticosterone / aCSF wash 15 min;
6. aCSF for 6 min;	6. 40mM ethanol for 6 min;	6. aCSF 6 min;	6. 40mM ethanol for 6 min;
7. 20μM NMDA for 6 min;	7. 20μM NMDA + 40mM ethanol for 6 min;	7. 20μM NMDA for 6 min;	7. 20μM NMDA + 40mM ethanol for 6 min;
8. 50μM NMDA for 6 min;	8. 50μM NMDA + 40mM ethanol for 6 min;	8. 50μM NMDA for 6 min;	8. 50μM NMDA + 40mM ethanol for 6 min;
9. 15 min aCSF wash;	9. 15 min aCSF wash;	9. 15 min corticosterone / aCSF wash;	9. 15 min corticosterone / aCSF wash;
10. 6min aCSF;	10. 80mM ethanol for 6 min;	10. 6 min aCSF;	10. 80mM ethanol for 6 min ;
11. 20μM NMDA for 6 min;	11. 20μM NMDA + 80mM ethanol for 6 min;	11. 20μM NMDA for 6 min;	11. 20μM NMDA + 80mM ethanol for 6 min;
12. 50μM NMDA for 6 min.	12. 50μM NMDA + 80mM ethanol for 6 min.	12. 50μM NMDA for 6 min	12. 50μM NMDA + 80mM ethanol for 6 min.

* = (corticosterone was present in the aCSF for the rest of the experiment including NMDA doses and wash periods);

The last one minute of all drug and washout periods was analysed as in the acute study.

Liquid diet treatment

Male TO mice (starting weight 25 –30g) were housed in groups of six under standard laboratory conditions (20°C and 40 - 60% humidity) and provided with all their calories as a liquid diet. Water was provided *ad libitum*. Chronically ethanol exposed animals were given diet containing ethanol in progressively increasing concentrations over 23 days (Lieber and De Carli, 1973).

Number of days	Ethanol content of diet
3	0 %
2	3.5 %
9	5.0 %
9	7.0 %

(alcohol intake values on page 244).

Daily volume consumed per cage was measured so that parallel control cages could be pair-fed the same amount of ethanol free diet (balanced for the caloric value of the ethanol). This was a crucially important step since the ethanol containing diet was never fully consumed (limited by either intoxication level or taste factors), whereas control animals would gorge on as much diet as was made available, potentially resulting in fatally obese control animals. Following the 23 days of liquid diet the diet was replaced by *ad libitum* access to standard dry pellet laboratory chow for 6 days. On day 7 after removal of liquid diet, animals were used for electrophysiological recordings.

Figure 5.4 a: A sample recording of the spontaneous activity of a dopamine sensitive cell of the ventral tegmental area.

Figure 5.4 b: An example of the action potential recorded from a dopamine sensitive cell of the ventral tegmental area.

Figure 5.4a: A sample trace recorded from a spontaneously active, dopamine sensitive cell from the Ventral Tegmental Area.

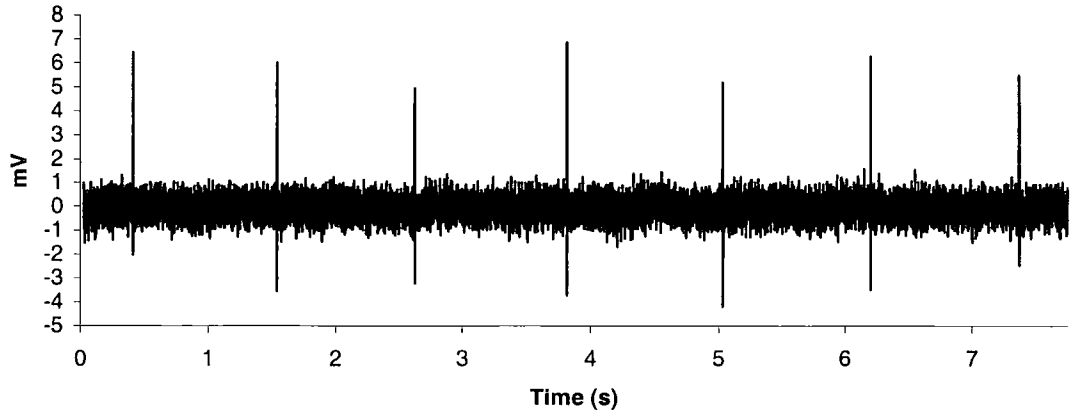
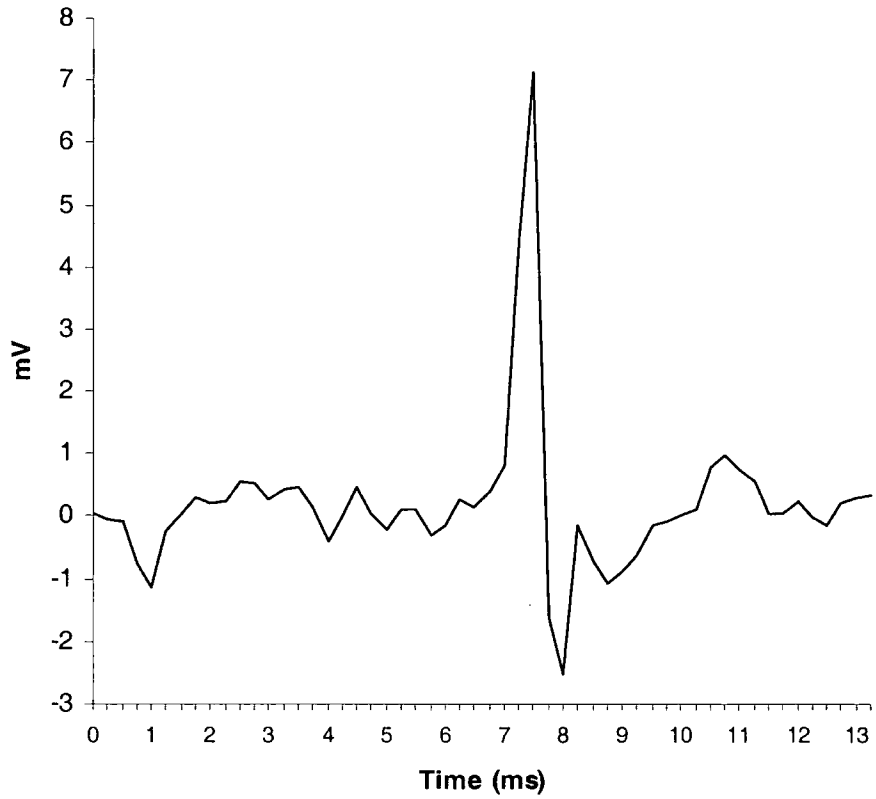


Figure 5.4b: An example of the action potential of the dopamine sensitive cells of the VTA



Results:

Extracellular recordings from dopaminergic cells of the VTA

Cells were selected for investigation based on action potential shape and frequency of firing as described in the method section. Dopamine (20uμM) decreased the firing rate of 100% of the neurons meeting these criteria within 10 minutes of dopamine application (figure 5.5).

Dose response curves to NMDA and ethanol.

NMDA increased the firing rate of the dopaminergic neurons of the VTA in a dose dependent manner in slices prepared at 10.00am from animals housed on reverse and normal phase (figure 5.6). The highest dose (100μM) of NMDA elevated the firing rate to such an extent as to prevent further firing, only 3 of 8 cells recovered following a protracted (25 minute) washout of NMDA in the normal phase animals. Interestingly there was no significant difference between the increase in firing rate of slices taken from animals during the reverse and normal phase at any NMDA concentration used. There was no significant effect of 200nM corticosterone on the effect of NMDA on firing rate in slices taken from reverse phase animals (figure 5.7).

Ethanol dose dependently increased the firing rate of VTA neurons from slices prepared at 10.00 am from animals housed on normal phase (figure 5.8).

Figure 5.5: The time course of action of 20μM dopamine in the perfusion medium on the firing rate of the spontaneously active dopamine cells of the ventral tegmental area slice from TO mice.

Figure 5.5: The timecourse of action of 20μM dopamine on the firing rate of VTA neurons.

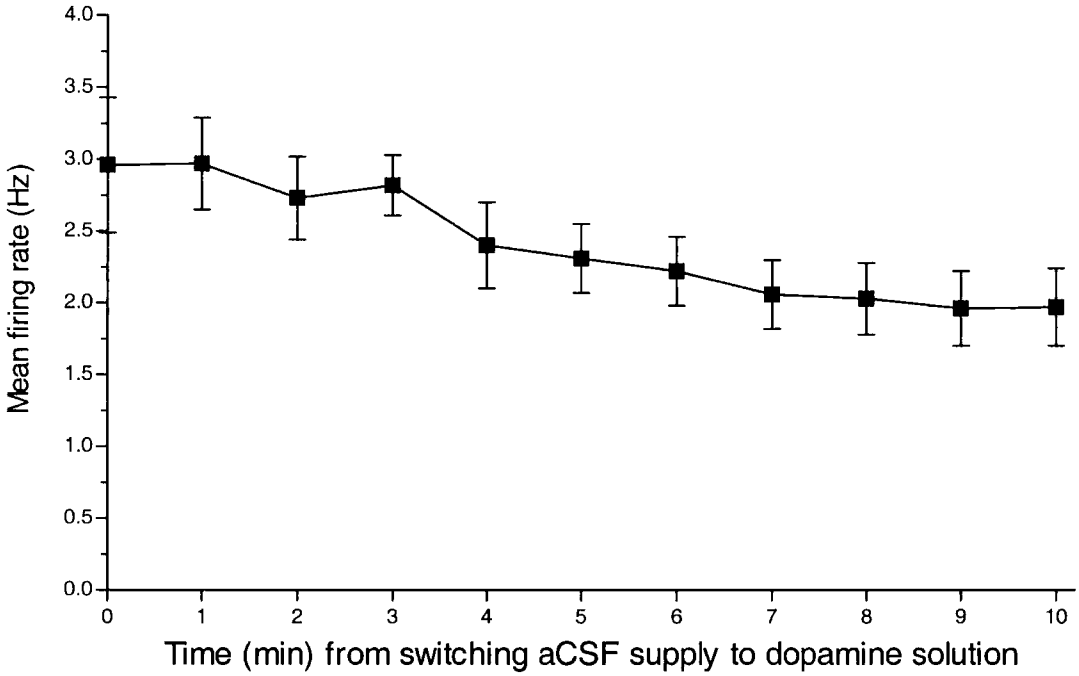


Figure 5.6: (a)The effect of 5, 20 50 and 100μM NMDA on the firing rate of spontaneously active dopamine neurons of the ventral tegmental area slice from TO mice. Slices were prepared at 10am from animals housed on reverse and normal phase and the effect of the phase of the light cycle at the time of slice preparation was investigated. Solid bars represent slices prepared at 10am from normal phase animals and diagonal hatched bars represent slices prepared at 10am from reverse phase animals. Raw data traces show baseline firing rate (Fig, 5.6b) and firing rate in the presence of 50μM NMDA (Fig. 5.6c).

Figure 5.7: The effect of continuous perfusion of 200nM corticosterone (CORT) on the increase in VTA cell firing induced by 5, 20, 50 and 100 μM NMDA. Midbrain slices containing the VTA were made from TO mice housed on normal phase. Vertical checked bars represent NMDA only controls, solid bars represents corticosterone and NMDA application.

Figure 5.6: The effect of NMDA on the firing rate of VTA neurons from TO mice housed on normal and reverse phase.

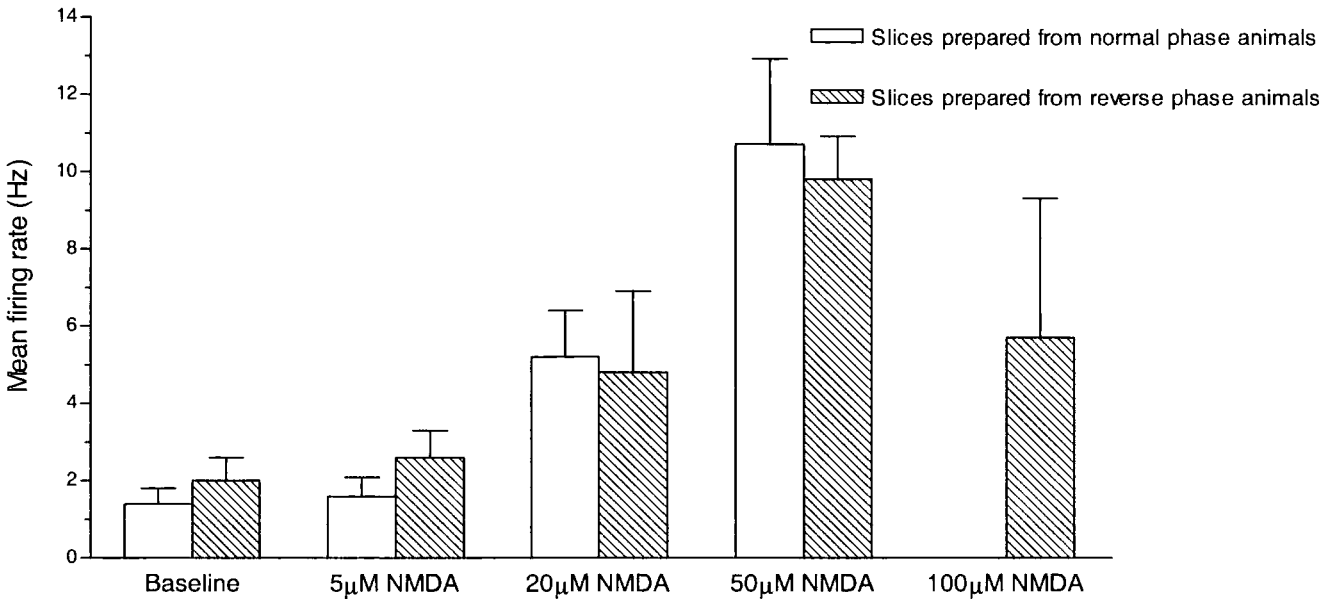
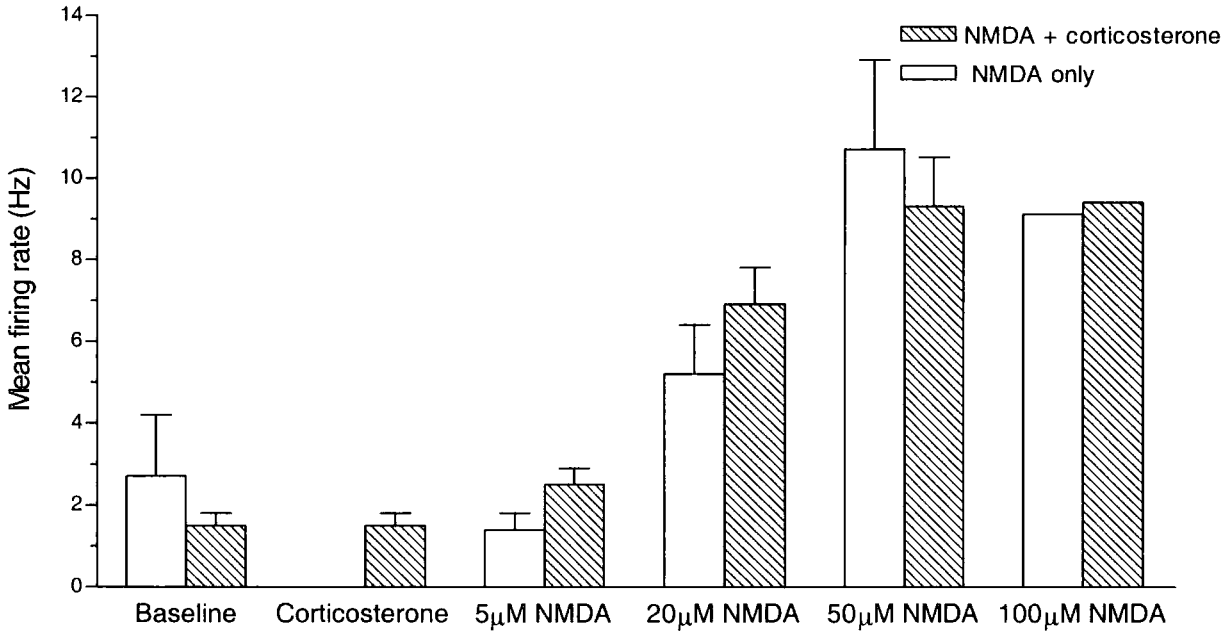


Figure 5.7: The effect of 200nM corticosterone on NMDA-induced increase in the firing rate of VTA neurons .



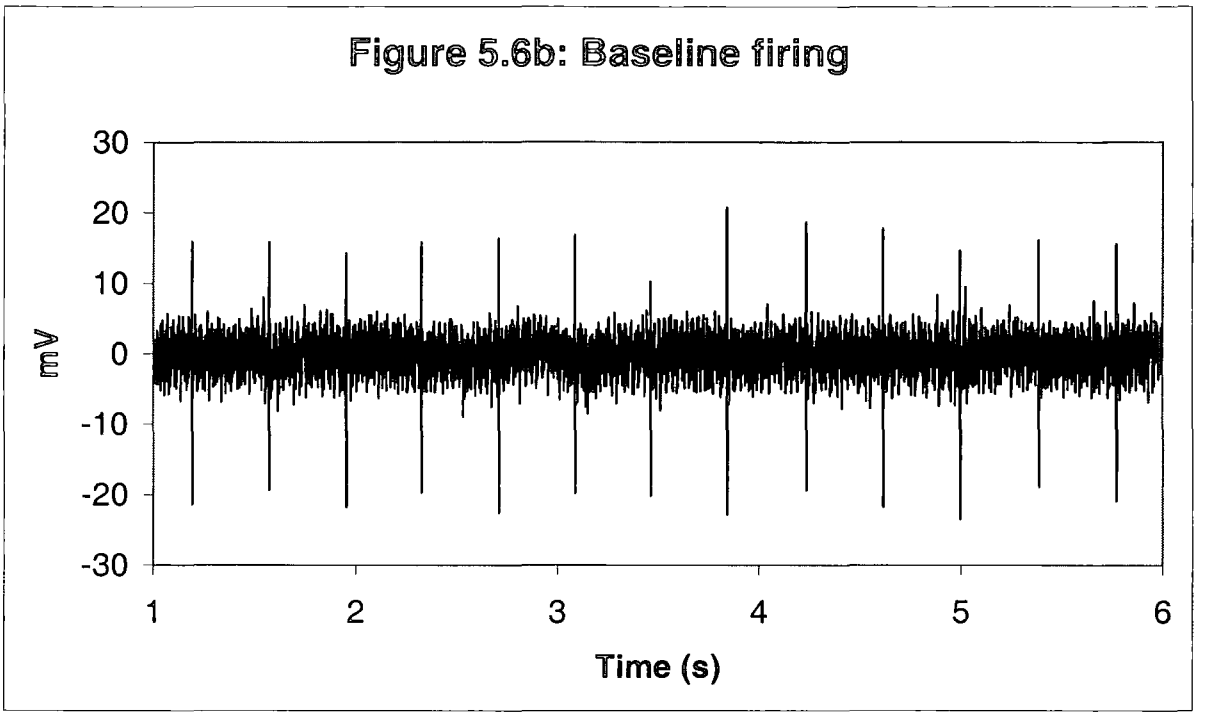


Figure 5.6b: Baseline firing
30
20
10
0
-10
-20
-30
mV
1
2
3
4
5
6
Time (s)

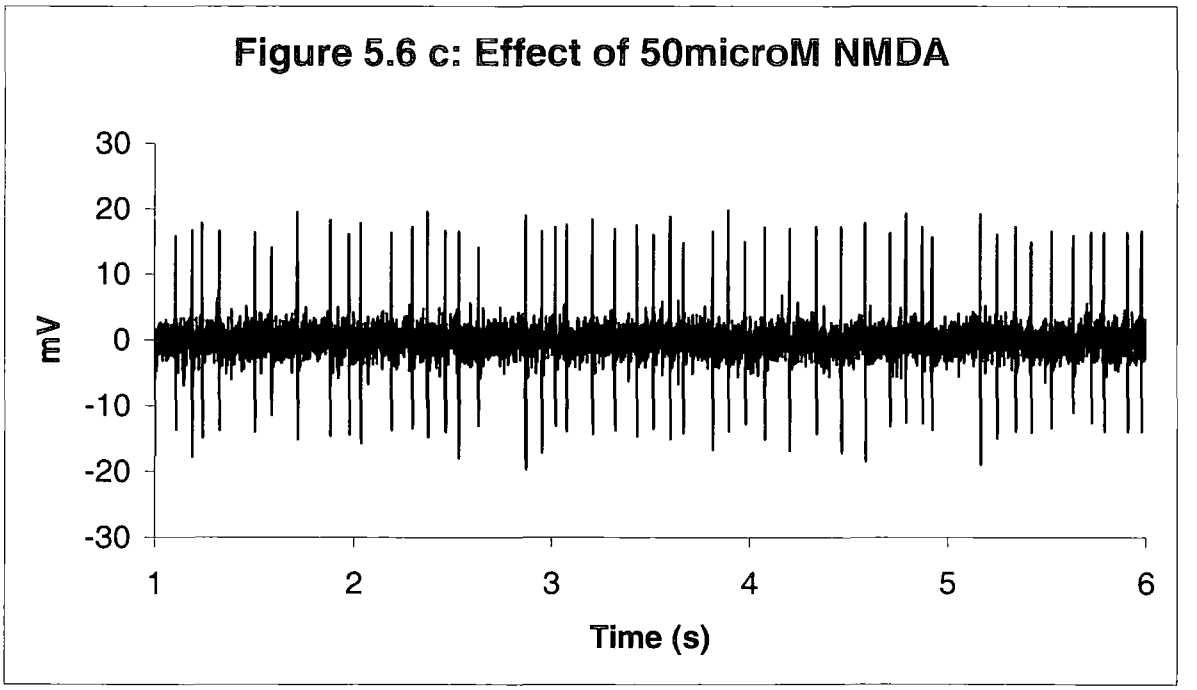


Figure 5.6 c: Effect of 50microM NMDA
30
20
10
0
-10
-20
-30
mV
1
2
3
4
5
6
Time (s)

Figure 5.8: The effect of acutely applied ethanol in the perfusion medium (40, 80, 160 and 320mM) on the firing rate of the dopamine cells of the VTA from TO mice (n=9). Raw data trace showing baseline firing (Fig. 5.8b) and firing rate in the presence of 160mM ethanol (Fig. 5.8c).

Figure 5.8a: The effect of ethanol on the firing rate of VTA neurons of reverse phase TO mice.

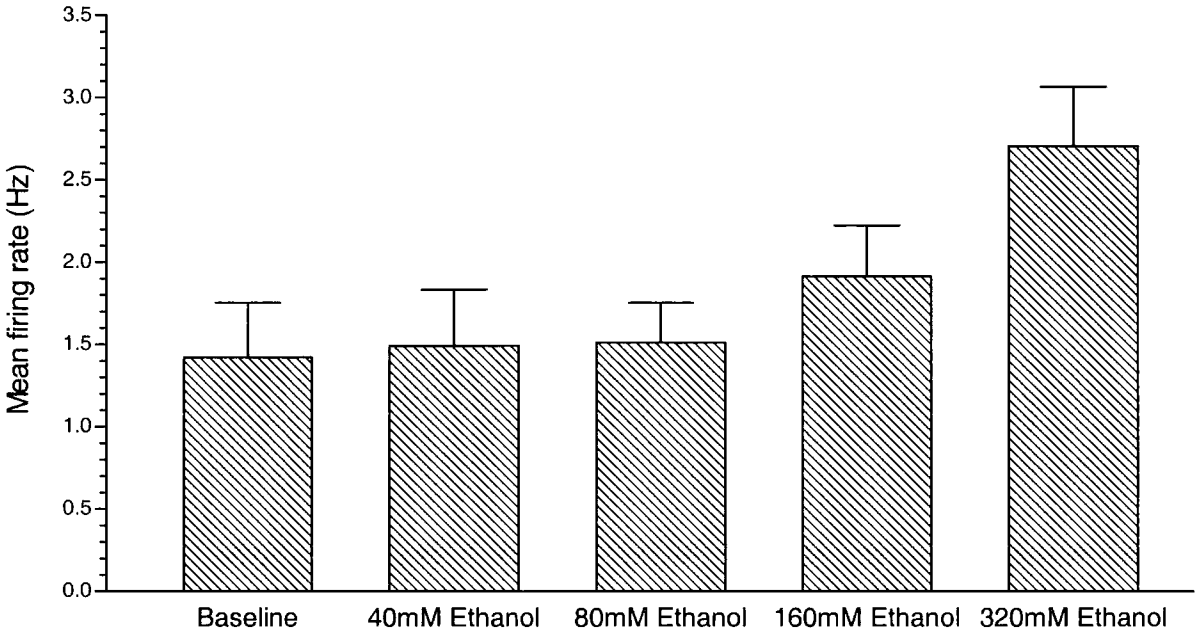


Figure 5.8b: Baseline Firing Rate

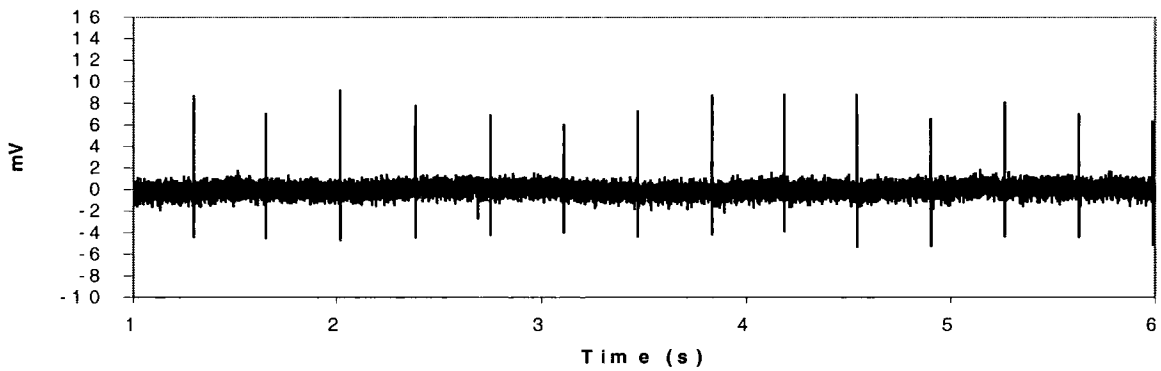
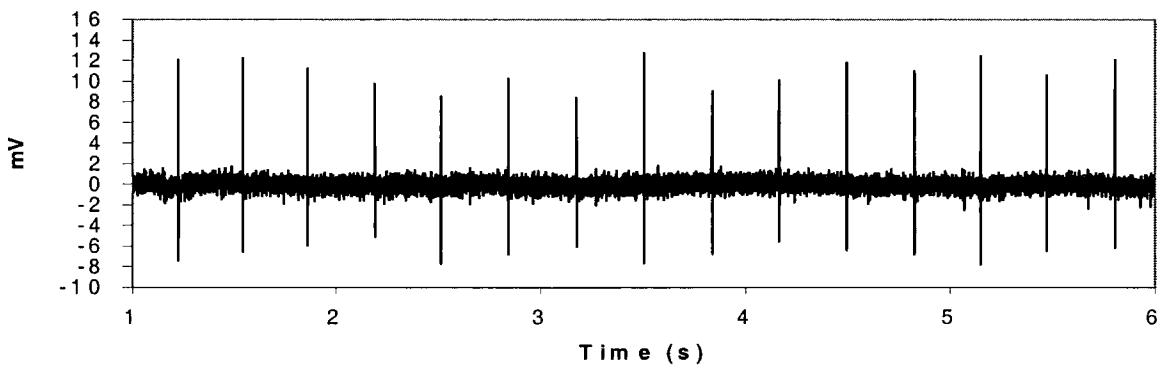


Figure 5.8 c: The effect of 160mM ethanol



Ethanol Intake as Liquid Diet

Cages of six animals consumed between 70 – 80 ml of diet each day, and the calorie - matched controls received a similar amount of diet each day. This level of diet intake resulted in an ethanol intake of 26 - 30 g/kg/24h. In fact, it was noted that during the first 24 h of the withdrawal phase several animals were slow and thin and seemed to be suffering from a fairly severe withdrawal syndrome, although no overt signs of withdrawal seizures were observed. Measurement of the withdrawal syndrome was not carried out as this has been clearly demonstrated by previous researchers in the lab using this specific diet regimen with group housed TO mice, although no loss of body weight or 'illness' was observed by previous researchers.

The effect of chronic ethanol intake on the baseline firing rate of the VTA

Six days after withdrawal from ethanol diet, there was no significant difference between the baseline firing rate of the VTA neurons from control (mean 1.7 ± 0.2 Hz, n=19) and ethanol treated mice (mean 1.6 ± 0.2 Hz, n=19). In order to control for variability in the baseline firing rate, all subsequent analysis was carried out on the percentage change in the firing rate of each cell relative to its own baseline firing rate rather than the mean firing rate for each condition.

The effect of chronic ethanol intake on NMDA and ethanol induced stimulation of VTA firing rate

As predicted by the results from the study of the acute effects of NMDA, 20uM and 50uM NMDA significantly increased the firing rate of the VTA neurons ($P< 0.005$). There was no significant difference between the increase in firing rate induced by the first second or third administration of NMDA at either concentration.

Ethanol pretreatment had no significant effect on the increase in firing rate induced by either 20 or 50μM NMDA (figure 5.9).

Due to the experimental difficulties mentioned previously, the group size for the 'ethanol and NMDA' test group was only 3 for animals fed control diet and 4 for animals fed ethanol diet. These n values are far too small for any meaningful statistical analysis to be carried out, however, the data for these groups is presented graphically in figure 5.10.

The application of 200nM corticosterone in the aCSF had no significant effect on the baseline firing rate of VTA slices from either ethanol pretreated or control animals. The effect of 20μM or 50μM NMDA application was not affected by the presence of 200nM corticosterone in the bathing medium for slices from either ethanol pretreated or control animals (figure 5.11).

The group size for the 'corticosterone, ethanol and NMDA' test group was 6 for animals fed control diet and only 4 for animals fed ethanol diet. The n value for the ethanol diet group is too small for statistical comparison with the control diet group, the data is presented graphically in figure 5.12.

Figure 5.9: The effect of repeated administration of 20 and 50μM NMDA on the firing rate of VTA dopamine cells after control (n=8; diagonal hatched bars) or ethanol diet (n=8; solid bars).

Figure 5.10: The effect chronic ethanol intake on the effects of ethanol (40 and 80mM) on NMDA (20 and 50μM) -induced firing of the dopamine cells of the VTA. Control diet (n=3) is represented by the diagonal hatched bars. Ethanol diet (n=4) is represented by the solid bars.

Figure 5.9: The effect of repeated NMDA exposure on NMDA-induced increases in the firing rate of VTA neurons of TOmice with and without ethanol experience.

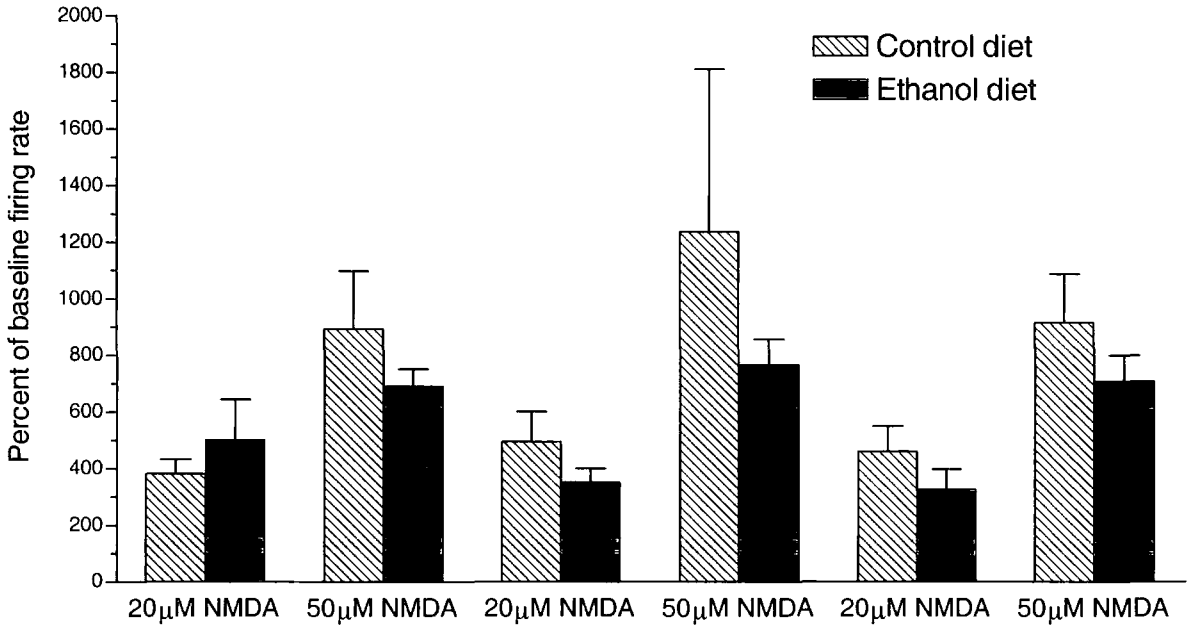


Figure 5.10: The effect of ethanol on baseline firing and NMDA-induced increases in the firing rate of VTA neurons of TOmice with and without ethanol experience.

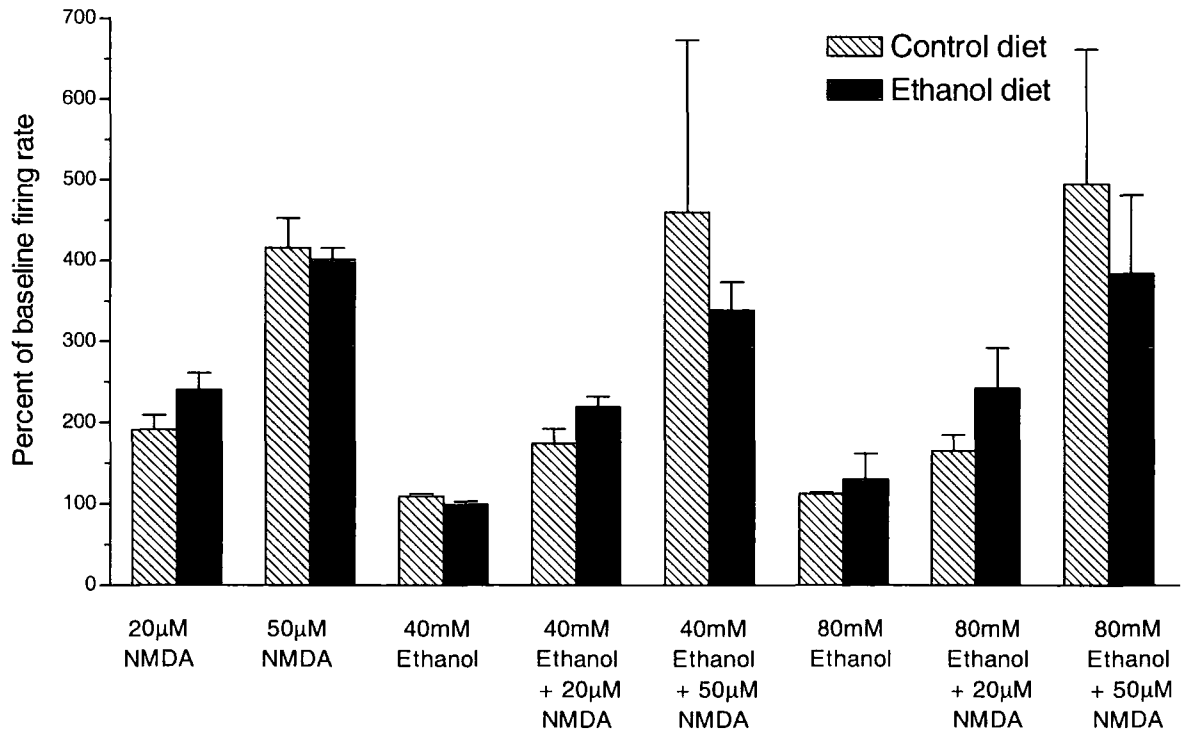


Figure 5.11: The effect of chronic ethanol intake on modulation of NMDA-induced dopamine cell firing in the VTA by corticosterone. Control diet (n=6) is represented by the diagonal hatched bars. Ethanol diet (n=7) is represented by the solid bars.

Figure 5.12: The effect of chronic ethanol intake on the effect of co-administration of ethanol (40 and 80mM) and corticosterone (200nM) on NMDA (20 and 50μM) – induced increases in the firing rate of the dopamine cells of the VTA. Control diet (N=4 is represented by diagonal hatched bars. Ethanol diet (n= 6) is represented by solid bars.

Figure 5.11: The effect of 200nM corticosterone on NMDA-induced increases in the firing rate of VTA neurons of TO mice with and without ethanol experience.

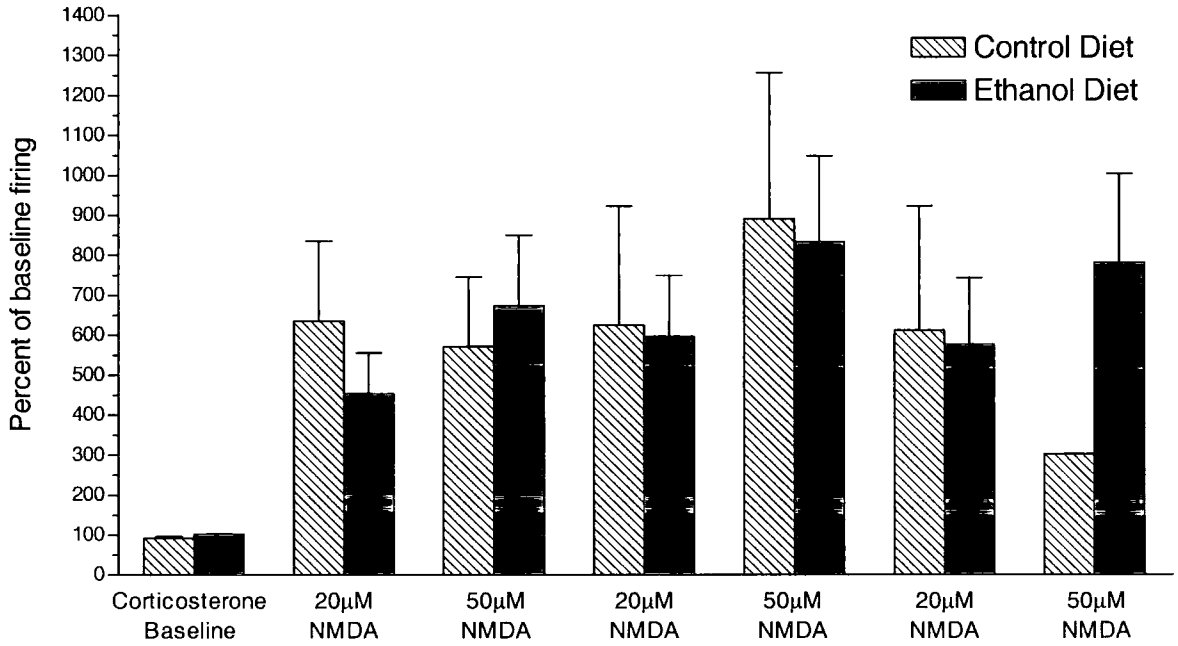
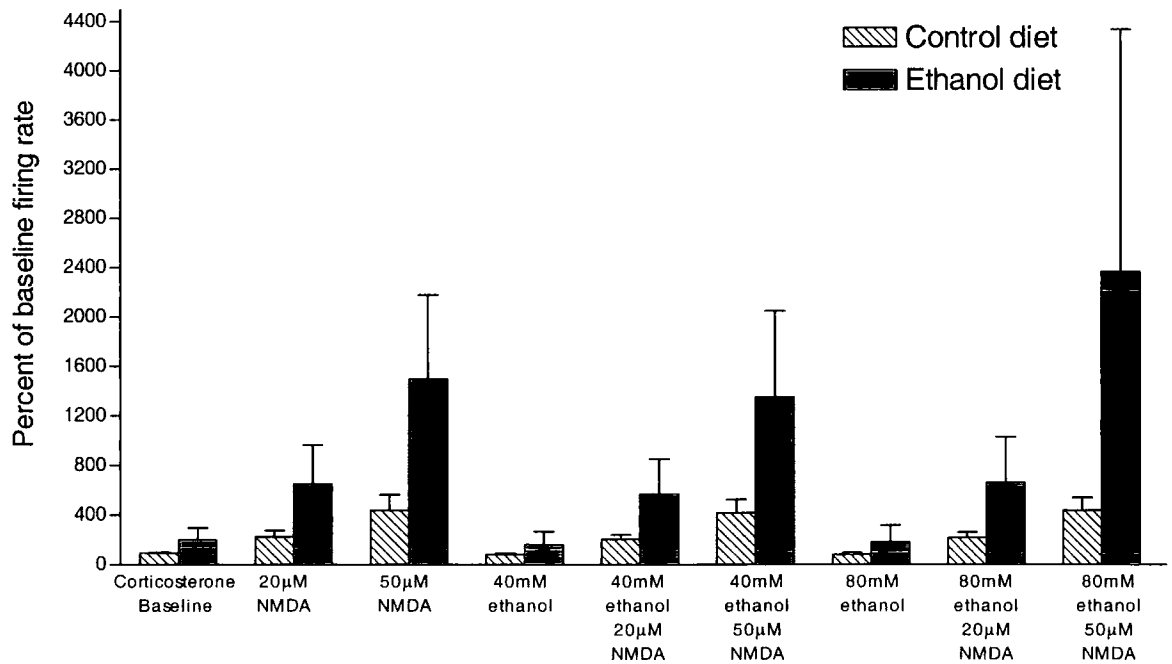


Figure 5.12: The effect of chronic ethanol intake on the effects of corticosterone and ethanol co-administration on NMDA-induced increases in the firing rate of VTA neurons of TO mice.



Discussion:

The effect of time of slice preparation and corticosterone on responses to NMDA

The application of 200nM corticosterone to the VTA slice had no effect on the basal firing rate of the dopaminergic neurons. This result was also reported by Cho and Little (1999) in a similar slice preparation. However, the study of Overton *et al.* (1996) using Sprague-Dawley rats under chloral hydrate anaesthesia, and investigating the effect of adrenalectomy, sham operation, and adrenalectomy with intravenous corticosterone replacement (13.4μg/100ml plasma) on basal firing and glutamate stimulated firing of the VTA, demonstrated a clear decrease in firing rate after adrenalectomy which was reversed with corticosterone replacement and a decreased sensitivity to glutamate. A result not seen in the isolated slice in my study or that of Cho and Little (1999). However Overton *et al.* (1996) essentially replaced the corticosterone after the removal of all circulating corticosterone, whereas Cho and Little (1999) and this study applied corticosterone at high concentrations on top of any corticosterone remaining in the slice and in addition to any prolonged genomic effects of the corticosterone present prior to decapitation.

NMDA stimulated an increase in firing rate of the VTA neurons in a dose dependent manner and this NMDA-induced increase in firing rate was not affected by the time of slice preparation or the co-application of 200nM corticosterone. This is contrary to the findings of Cho and Little (1999) using mid brain slices from Wistar rats in this laboratory but not the findings of Overton *et al.* (1996) using *in vivo* recordings from adrenalectomised rats. In addition to the obvious species difference, Cho and Little (1999) did not use halothane anaesthesia prior to decapitation but instead used a schedule I concussion and dislocation technique. Whether the use of halothane has hidden the effect of the corticosterone, or the concussion / dislocation technique has affected the sensitivity to NMDA and corticosterone is unclear at this point, however.

Iontophoretic application of glutamate increased the firing rate of the VTA neurons *in vivo*, however there was no effect of adrenalectomy or corticosterone replacement on this glutamate-induced increase in basal firing rate of these cells (Overton *et al.*, 1996) – in agreement with my results and contrast to those of Cho and Little (1999). There was a significant effect of glutamate application and corticosterone status on the burst firing activity of cells classed as 'bursting' (based on clustering of 3 or more spikes in 500 consecutive spikes) but not those classed as 'non-bursting' (Overton *et al.*, 1996).

The results of Cho and Little (1999) directly contrast my results showing no effect of corticosterone on NMDA stimulated VTA firing in the slice environment using, essentially, local application of corticosterone to the slice. However, the findings of Overton *et al.* (1996) *in vivo* demonstrate a similar lack of effect of systemic corticosterone on glutamate stimulated basal VTA firing, but a significant effect of corticosterone or adrenalectomy on basal firing rate. The more intricate results reported by Overton *et al.* (1996) involving the interplay of corticosterone and glutamate actions on burst firing activity indicate that there may be a role for corticosterone in the modulation of glutamate-stimulated VTA firing (as supported by Cho and Little, 1999). However, this role is probably a very subtle and complicated one requiring study utilising the complete neuronal networks of the intact animal to truly clarify the effects of corticosterone rather than the slice environment. A crucial aspect of the work of Overton *et al.*, (1996) as compared to that of Cho and Little (1999) was that Overton *et al.*, (1996) made recordings in adrenalectomised animals with little or no circulating corticosterone, whereas Cho and Little (1999) simply applied corticosterone to slices taken from intact (un-adrenalectomised) animals. Thus Cho and Little were looking for effect of high corticosterone concentrations (100nM) on top of any remaining corticosterone occupying intracellular receptors or any lasting genomic effects of corticosterone.

Along with the lack of effect of directly applied corticosterone, there was no difference in the NMDA sensitivity of the VTA taken from mice during their light and dark phases (times of low and high circulating corticosterone respectively). These two sets of results agree on a lack of corticosterone sensitivity of the basal firing of VTA slices prepared from TO mice in the manner used here.

The effect of ethanol on VTA firing rate

The dose dependent stimulation of VTA firing by ethanol is a fairly robust effect that has previously been reported by Brodie *et al.* (1990, 1999) in a similar range of concentrations using rats. An important point to note, however, when considering the results from this study (and that of Brodie *et al.*, 1990, 1999) is that the range of ethanol concentrations used are on the higher end of a physiologically relevant range. Indeed, 40mM would be indicative of a moderate level of intoxication and not the lowest dose to produce pharmacological effects in whole animals or humans. However, a preliminary study indicated that lower ethanol concentrations (20mM) did not produce consistent effects. Out of 6 neurons tested, 2 showed an increased firing rate over baseline (1 and 5% increases), while three showed very small decreases in firing rate compared with baseline (2, 3 and 6%) and the firing of one cell was decreased by 22% of the baseline rate. Since the effect of 20mM ethanol was very small and unreliable, the lowest concentrations used in the dose response curve experiment was 40mM.

Although the data set produced was small, there also appeared to be no interaction between ethanol and NMDA –induced increases in firing rate. This has not been investigated previously and, while the result may demonstrate that ethanol does not interact with NMDA the data set is rather small for true conclusions to be drawn.

The effect of withdrawal from chronic ethanol on the firing rate of VTA cells

The baseline firing rate of the VTA cells was not significantly different between animals that had been pretreated with ethanol and ethanol naïve controls. Sufficient ethanol containing diet had been consumed by individuals to produce clear signs of a withdrawal phase, as documented previously by Bailey *et al.* (1998), in a similar study of basal VTA firing after 6 days abstinence. However, the mean firing rate in this study was the same as the 'lower' firing rate described by Bailey *et al.*, (1998) after ethanol diet and in the presence of 5μM NMDA. These authors reported a control firing rate of 3.4 ± 0.7 Hz in slices from ethanol naïve animals, considerably higher than the 1.7 ± 0.2 Hz reported here. Interestingly, the study of Bailey *et al.* (2000), similar to that of Cho and Little (1999), did not use halothane anaesthesia prior to decapitation but rather a schedule I dislocation procedure. This may account for the lack of difference in the baseline firing rate following ethanol pretreatment and the apparent depression of the firing rate of control animals. However, it seems a little unlikely since both control and ethanol pretreated animals were exposed to halothane, and then only very briefly and Bailey *et al.* (1998) 'artificially' elevated the firing rate of the ethanol treated group with 5μM NMDA to achieve the same firing rate as reported here. Also, over-exposure to halothane decreased the firing rate of cells to the point of the slice being considered unusable even with the low level NMDA stimulation used by Bailey *et al.* (1998) with mice that had received chronic ethanol treatment. One would imagine that were a low level of halothane to compensate for a decrease in baseline firing rate induced by withdrawal from chronic ethanol then a higher dose would not necessarily decrease the firing rate to the point of being undetectable. However, a biphasic effect of halothane – stimulating firing in withdrawn animals at low (yet anaesthetic) doses, but inhibiting firing in higher doses can not be ruled out.

During the original setup of the procedures to be used for the electrophysiological study, dislocation and decapitation was the originally planned method of killing the animals, however, dislocation was not a very stable method in my hands as I could not reliably avoid a build up of blood in the meninges at the base of the skull and brain resulting in slices without any signs of spontaneous activity, possibly due to damaged tissues from the increase in cranial blood pressure directly prior to death. Following these concerns, and the reliability and ease of use of halothane to produce spontaneously active slices, the 'light' anaesthesia method was chosen.

Following pretreatment with ethanol or control diet and six days abstinence neither ethanol nor corticosterone appeared (n value too low for statistical analysis) to affect the level of NMDA induced increases in firing rate of the VTA cells from ethanol pretreated or control animals. These results reiterate the lack of effect of corticosterone as demonstrated in the acute study. Also the lack of difference between control and ethanol pretreated animals that is displayed in the analysis of baseline firing is further supported by the lack of any differences in the effects of NMDA or corticosterone on the firing rate of VTA cells following either diet regimen.

Chapter 6

Summary and Conclusions

Summary and conclusions

The overall hypothesis for this thesis was that stress (primarily manifested as elevated corticosterone levels) plays a significant role in the regulation of ethanol consumption and the cellular actions of ethanol on the central reward pathway. The data presented in this thesis provides further insight into the complexity of the interaction between stress and ethanol and indicates a potential role for repeated high levels of stress in the modulation of ethanol consumption. However, these results do not provide for an unequivocal acceptance of dismissal of the hypothesis tested.

Investigations into the effect of social status or group housing on the ethanol consumption of rats or mice respectively showed no real link between low levels of social stress associated with establishment and maintenance of the social hierarchy or subordination. This seems contrary to the results of Blanchard *et al.*, (1992,1993,1995), although it is unclear whether this difference is a result of the standardized laboratory environment used in this thesis as compared to the visible burrow system of Blanchard *et al.*, (1992,1993,1995).

In order to further pursue the issue of social stress and ethanol intake, a resident-intruder paradigm was used to investigate the effect of aggressive defeat on the ethanol intake of a 'stress sensitive' strain of mouse (C57). The results from this study indicated that repeated social defeat had a significant effect on ethanol intake, but a single defeat had no more effect than the novelty control (with no aggressive animal present). Thus it seems that, while social stress may play a role in elevated ethanol consumption, a single event is not sufficient to precipitate a prolonged elevation in ethanol intake when examined under controlled laboratory conditions. Whether a naturalistic environment provides for a greater degree of sensitivity to stressful events can not be deduced from these, or earlier studies by other authors.

These results offer no information as to whether a single stressful event is sufficient to induce relapse in rodents as reported with human patients, although the reinstatement of operant self-administration of ethanol can be stimulated with a single, high stress situation (Le *et al.*, 1999).

These results do indicate a significant effect of repeated social defeat on the ethanol intake of C57 mice that occurs over a far longer time course than any previously documented effects of social defeat (Miczek *et al.*, 1991; Tornatzky and Miczek, 1993; Krugers *et al.* 1993; Korte *et al.*, 1995; Tidey and Miczek, 1996). The next step would be to investigate the mechanism of action of social defeat on ethanol intake. It could be postulated that exposure to novelty would activate the HPA axis in a classical stress response and that the resultant elevated levels of circulating corticosterone and CRF seen with HPA activation would stimulate the transient increase in ethanol intake.

Repeated social defeat has been shown to have lasting effects on the circadian regulation of core body temperature and heart rate in Long Evans rats for 10 - 12 days following defeat (Tornatzky and Mizcek, 1993; Harper *et al.*, 1996) and since corticosterone production also follows a circadian rhythm it would seem reasonable to suggest that this rhythm may also be disrupted by social defeat. The C57BL/10 strain of mice used showed elevated ethanol intake several weeks after repeated saline injections (Little *et al.*, 1999) and on exposure to novelty in this study.

Le *et al.* (1998, 1999, 2000) demonstrated that footshock stress and CRF injections, but not corticosterone injections (Le *et al.*, 2000), reinstated operant ethanol self-administration, and Fahlke *et al.* (1994a, b) have demonstrated that removal of circulating corticosterone by adrenalectomy or by the inhibition of corticosterone synthesis with metyrapone will decrease ethanol intake. This would

tend to suggest that a long term disruption in the circadian rhythm of the HPA axis could result in increased ethanol intake, if circulating corticosterone and/or CRF was increased as a result. Sardinian alcohol-preferring rats have been shown to consume ethanol with a distinct circadian rhythm involving three periods of drinking that coincided with food intake periods (Agabio, *et al.*, 1996). The results presented here indicate that, whilst exposure to a novel cage will raise ethanol intake transiently, 5 daily social defeats may be required to produce a long term disruption to the circadian rhythm of the HPA axis.

It should be clarified that these conclusions, with respect to disruption of the circadian rhythm of corticosterone and CRF production, are not proved directly by the results from this study. However, these results add to the steadily increasing volume of results indicating a role for social stress and the HPA system in the modulation of ethanol intake, including the clear effects of repeated defeat on the circadian rhythms of rat homeostatic mechanisms, and the important role of CRF (and corticosterone) in ethanol consumption.

Curiously, however, the preliminary electrophysiology data presented showed no effect of corticosterone on the baseline or stimulated firing rate of the dopamine cells of the ventral tegmental area. Nor was there any interaction between ethanol and corticosterone on the firing of these of the central dopamine reward pathway. Thus the hypothesis that ethanol dependence and consumption is modulated at least in part by stress, via corticosterone, can not be fully supported by these findings. Repeated stress appears to play a significant role in long term changes in ethanol consumption, but corticosterone itself appears to have no effect on the cellular activity of the central reward systems.

Further investigations are necessary to clarify the role of stress and the HPA axis in modulating ethanol consumption would. Some specific points of interest would be:

1) confirmation that repeated defeat in the resident intruder paradigm following 3 weeks access to ethanol actually produces an elevation in corticosterone production, and how long this elevation remains.
2) investigation into the sensitivity of the mesolimbic reward pathway to ethanol after repeated defeat in light of the altered ethanol preference after repeated but not single defeat.
3) verification of the interaction between corticosterone and NMDA on the firing rate of VTA cells using brain slices from adrenalectomised animals. This would also allow for investigation of the role of the mineralocorticoid receptor system in this interaction.

Further studies could also investigate drug interventions that would block the defeat induced increase in ethanol preference, some candidates of interest might be: the dihydropyridine calcium channel blockers (although the decreased preference previously reported would make it unclear whether they were blocking the stress response or just decreasing preference in general); CCK_B antagonists, which have been shown to prevent the i.p. injection induced increases in ethanol preference (Little *et al.*, 1999); upstream blockade of the HPA response using CRF antagonists; commonly used alcoholism treatments that are thought to help prevent relapse such as naltrexone or acamprosate.

In conclusion, stress seems to be an important factor in ethanol consumption and relapse, however, whether this is an action of corticosterone itself and if so whether it is acting directly on the dopamine reward system or through interaction with the excitatory amino acid system still remains unclear.

Further conclusions of relevance to the field of ethanol dependence, but not directly pertinent to the original hypothesis tested, stem from the use of the 4 bottle choice model of ethanol access proposed by Wolffgramm and Heyne (1995). The data from two groups of animals provided with four bottle choice ethanol access is presented in this thesis and it appears that this model of ethanol access does not demonstrate many of the aspects of ethanol dependence required for a new model of ethanol. No increase in ethanol consumption, switch in preferred concentration or any other sign of loss of control over ethanol consumption was observed while the age of the animals is a major confounding factor without any benefits over the traditional two bottle preference model. Of possible interest is the lack of effect of the dihydropyridine calcium channel antagonist, nimodipine, on ethanol consumption in this model. However, in light of the small volumes of ethanol consumed in this model, the lack of effect of nimodipine may not be a result with any great significance in the development of therapeutic interventions for ethanol dependence.

Appendix:

Diagnostic and Statistical Manual of Mental Disorders IV – Criteria for Substance abuse and dependence.

Substance Abuse

A: A maladaptive pattern of substance use leading to clinically significant impairment or distress, as manifested by one (or more) of the following, occurring within a 12-month period:

- recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home (e.g., repeated absences or poor work performance related to substance use; substance-related absences, suspensions or expulsions from school; neglect of children or household).
- recurrent substance use in situations in which it is physically hazardous (e.g., driving an automobile or operating a machine when impaired by substance use).
- recurrent substance-related legal problems (e.g., arrests for substance-related disorderly conduct).
- continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance (e.g., arguments with spouse about consequences of intoxication, physical fights).

B: The symptoms have never met the criteria for Substance Dependence for this class of substance.

Substance Dependence

A maladaptive pattern of substance use leading to clinically significant impairment of distress, as manifested by three (or more) of the following, occurring at any time in the same 12-month period:

1. Tolerance, as defined by either of the following:

    a. a need for markedly increased amounts of the substance to achieve intoxication

    b. markedly diminished effect with continued use of the same amount of the substance

2. Withdrawal, as manifested by either of the following:

    a. the characteristic withdrawal syndrome for the substance

    b. the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms

3. The substance is often taken in larger amounts or over a longer period than was intended

4. There is a persistent desire or unsuccessful efforts to cut down or control substance use

5. A great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors or driving long distances), use the substance (e.g., chain-smoking), or recover from its effects

6. Important social, occupational, or recreational activities are given up or reduced because of substance use

7. The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance (e.g., current cocaine use despite recognition of

cocaine-induced depression, or continued drinking despite recognition that an ulcer was made worse by alcohol consumption).

The ICD-10 (International Classification of Diseases) Classification of mental and behavioural disorders

Diagnostic Criteria for Clinical Use:

Harmful Use:

A pattern of psychoactive substance use that is causing damage to health. The damage may be physical (as in cases of hepatitis from the self-administrationof injected drugs) or mental (e.g., episodes of depressive disorder secondary to heavy consumption of alcohol). The diagnosis requires that actual damage should have been caused to the mental or physical health of the user. Harmful patterns of use are often criticized by others and frequently associated with adverse social consequences of various kinds. The fact that a pattern of use of a particular substance is disapproved of by another person or by the culture, or may have led to socially negative consequences such as arrest or marital arguments is not in itself evidence of harmful use. Acute intoxication or "hangover" is not in itself sufficient evidence of the damage to health required for coding harmful use. Harmful use should not be diagnosed if dependence syndrome, a psychotic disorder, or another specific form of drug- or alcohol-related disorder is present.

Dependence Syndrome

A definite diagnosis of dependence should usually be made only if three or more of the following have been experienced or exhibited at some time during the previous year:

1. A strong desire or sense of compulsion to take the substance.

2. Difficulties in controlling substance-taking behavior in terms of its onset, termination, or levels of use.
3. A physiological withdrawal state when substance use has ceased or been reduced, as evidenced by: the characteristic withdrawal syndrome for the substance; or use of the same (or closely related) substance with the intention of relieving or avoiding withdrawal symptoms.
4. Evidence of tolerance such that increased doses of the psychoactive substance are required in order to achieve effects originally produced by lower doses (clear examples of this are found in alcohol- and opiate-dependent individuals who may take daily doses sufficient to incapacitate or kill nontolerant users).
5. Progressive neglect of alternative pleasures or interests because of psychoactive substance use, increased amounts of time necessary to obtain or take the substance or recover from its effects.
6. Persisting with substance use despite clear evidence of overtly harmful consequences, such as harm to the liver through excessive drinking, depressive mood states consequent to periods of heavy substance use, or drug-related impairment of cognitive functioning; efforts should be made to determine that the user was actually, or could be expected to be, aware of the nature and extent of harm.

Narrowing of the personal repertoire of patterns of psychoactive substance use has also been described as a characteristic feature (e.g., a tendency to drink alcoholic drinks in the same way on weekdays and weekends, regardless of social constraints that determine appropriate drinking behavior). It is an essential characteristic of the dependence syndrome that either psychoactive substance taking or a desire to take a

particular substance should be present; the subjective awareness of compulsion to use drugs is most commonly seen during attempts to stop or control substance use. This diagnostic requirement would exclude, for instance, surgical patients given opioid drugs for the relief of pain, who may show signs of an opioid withdrawal state when drugs are not given but who have no desire to continue taking drugs. The dependence syndrome may be present for a specific substance (e.g., tobacco or diazepam). for a class of substances (e.g., opioid drugs), or for a wider ranger of different substances (as for those individuals who feel a sense of compulsion regularly to use whatever drugs are available and who show distress, agitation, and/or physical signs of a withdrawal state upon abstinence). The diagnosis of the dependence syndrome may be further specified by the following (the following roughly correspond to the course modifiers and relapse section of DSM-IV):

- Currently abstinent.
- Currently abstinent, but in a protected environment (e. g., in hospital, in a therapeutic community, in prison, etc.).
- Currently on a clinically supervised maintenance or replacement regime (controlled dependence, e.g., with methadone; nicotine gum or nicotine patch).
- Currently abstinent, but receiving treatment with aversive or blocking drugs (e. g., naltrexone or disulfiram).
- Currently using the substance (active dependence).
- Continuous use.
- Episodic use.

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