Stress and neurochemical changes associated with chronic alcohol administration

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Statement of copyright

Declaration.

I hereby declare that this thesis has not been submitted, either in the same or different form, to this or any other University. I declare that the original work contained within this thesis I conducted, unless otherwise stated. The experiments performed in Chapter Four using *alpha-helical* corticotrophin releasing factor and corticotrophin on low alcohol-preferring mice was conducted in collaboration with Adam Croft.

Matthew Joseph O'Callaghan

Publications arising from the thesis

O'Callaghan, M. J., Croft, A., Brooks, S.J., Watson, W.P. and Little H.J. (2001) Low alcohol preference among the 'high alcohol preference' C57 strain of mice; studies on the factors affecting such preference. *In preparation.*

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Little, H.J., Butterworth, A.R., O'Callaghan, M.J., Wilson, J., Cole, J. and Watson, W.P. (1999) Low alcohol preference among the "high alcohol preference" C57 strain of mice: preference increased by saline injections. Psychopharmacol. **147:** 182 – 189.

O'Callaghan, M.J., Cole J.C. and Little H.J. (1999) Effects of vehicle injections on preference of C57 strain mice for alcohol. Br. J. Pharmacol. **127:** 32P.

O'Callaghan M.J. and Little, H.J. (1998) No correlation between ethanol preference and total plasma corticosterone in C57 strain mice. J. Psychopharmacol. **12:** 3, P39.

Thesis title:
Stress and neurochemical changes associated with chronic alcohol administration.

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Abstract: There is considerable recent experimental evidence that suggests that stress plays a major role in the development of dependence on drugs of abuse, but the potential mechanisms involved are not yet fully understood.

The aims of this thesis were (I) to examine the effect of stress, and of drugs that act on the hypothalamic-pituitary-adrenal axis, on alcohol consumption in the C57 strain of mice. (II) to investigate whether corticosterone levels or spontaneous locomotor activity could be used to predict subsequent alcohol consumption in mice. (III) to investigate long-term neurochemical changes during abstinence following chronic alcohol administration. (IV) to develop a method for measuring brain corticosterone levels.

Neither total corticosterone levels nor spontaneous locomotor activity could predict alcohol preference. Saline vehicle injections increased alcohol preference in low alcohol preferring, and raised both circulating corticosterone levels and brain corticosterone. The brain concentrations of corticosterone were measured by a novel procedure developed during the thesis. A CRF antagonist (α-helical CRF) increased alcohol preference in low preferring mice, as did the ACTH fragment 4-10. In high preferring mice, ACTH $_{4\text{-}10}$ reduced alcohol preference, whereas α-helical CRF did not alter preference in these mice. Inhibition of corticosterone synthesis reduced alcohol preference in high alcohol preferring mice but, blockade of corticosterone receptors with specific antagonists did not alter alcohol preference. Chronic alcohol treatment followed by six days abstinence increased free circulating corticosterone levels and this treatment also increased hippocampal corticosterone levels. Dopamine D1-like receptor affinity was increased following the same chronic treatment schedule. These results demonstrate an important link between the activation of the hypothalamic-pituitary-adrenal axis and alcohol consumption. The results of the chronic treatment experiments provide useful information that may aid the understanding of the phenomenon of relapse to drinking common in abstaining alcoholics.

Dedication:

To Fi and Dad

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Abbreviations

ACTH	adrenocorticotrophin hormone (a.k.a. corticotrophin)
AMPA	α-Amino, 3-hydroxy, 5-methyl, 4-isoxazolepropionic acid
CRF	corticotrophin releasing factor
CCK	cholecystokinin
DSM IV	Diagnostic and Statistical Manual of Mental Disorders (4^{th} edition)
GABA	γ–aminobutyric acid
HPA	hypothalamic- pituitary- adrenal
5HT	5-Hydroxytryptamine
i.p	intra peritoneal
i.c.v.	intra-cerebroventricular
MSH	melanocyte-stimulating hormone
NMDA	N-methyl D-aspartate
POMC	Pro-opiomelanocortin
VTA	ventral tegmental area

Contents

Chapter One

Introduction

Outline of the Thesis

The experiments described in this thesis were performed in order to understand the influence corticosterone and stress have on alcohol consumption. This work investigated long-term neuronal changes after alcohol consumption that might aid the explanation of the phenomenon of relapse drinking. The Thesis is divided into two main experimental sections; the first (Chapters 2-5 inclusive) examined the effects of stress (repetitive saline injections) and pharmacological interventions on the hypothalamic-pituitary adrenal (HPA) axis and on alcohol preference in studies performed on C57 mice, a strain of mice commonly used in alcohol research. The second section (Chapters 6 and 7) investigated potential long-term changes in neurochemistry after chronic alcohol administration. The methods used in this thesis are detailed in each chapter, rather than in a specific 'General Methods' section. All reagents and drugs were purchased from Sigma-Aldrich (U.K.) except where clearly described in the text.

The social and medical cost of alcohol

Alcohol is one of the most widely used drugs of abuse. The social, economic and health related costs that alcohol makes upon society are immense. The importance of understanding alcohol dependence and the effects of alcohol therefore cannot be underestimated when viewed in the context of the problems it causes, some of which are detailed below.

Alcohol related health problems have been estimated to cost the National Health Service (NHS) £150 million a year (Shakeshaft et al., 1997). In urban hospitals in Britain, 15-30% of male medical and surgical patients and 8-15% of women patients have alcohol problems, on the basis that they reported two or more problems related to psychological or physical dependence. (Chick et al 1993). It is estimated that 20% of the workload of GPs arises from the effects of heavy drinking, compounded by the problems which arise because alcohol abuse creates medical problems for family members (Kaner et al., 1997). In England, in 1995, there were 20,727 admissions to NHS hospitals for alcohol dependence and 2076 admissions for alcohol poisoning (Alcohol Alert 2000). Chronic alcoholism constitutes a significant public health problem; it is associated with a wide range of diseases and is a significant cause of premature death and persistent brain damage; such as Wernicke's encephalopathy, Korsakoff's syndrome, cerebellar degeneration and dementia (Alcohol Alert 2000). Excessive alcohol is implicated in reproductive (loss of libido, hormonal imbalances, foetal abnormalities), cerebrovascular, respiratory, liver, gastrointestinal, heart, and circulatory problems (Alcohol Alert 2000).

The 1987 report Royal College of Physicians: A Great and Growing Evil - The Medical Consequences of Alcohol Abuse has estimated mortality associated with alcohol consumption to be of the order of 5,000 - 8,000 a year. The number of deaths attributable to alcohol is a matter of controversy, as death certificates do not record alcohol as a contributory cause of death. Alcohol is a contributing factor in deaths from a range of other causes: - accidents, suicide, cancers and heart disease amongst others. It is estimated that there are 2 million people in Britain experiencing symptoms of alcohol dependence (Alcohol Alert

2000). The 1993 Health Survey for England classified 9% of men and 5% of women who drank as 'problem drinkers', i.e. exhibiting one or more the criteria for alcohol dependence or alcohol abuse. A third of road traffic accidents have alcohol reported as a contributing factor, and it is an important factor in domestic, recreational and work-related accidents (Petersson et al 1982). Family disruption, domestic violence, child abuse, public order problems and absenteeism from work are all consequences of alcohol abuse.

Dependence and addiction

The term 'addiction' is usually taken to mean "a state of physical and psychological dependence" .The World Health Organisation (WHO) recommended that the term 'addiction' should be replaced by the term dependence. In 1974 the WHO defined drug dependence as 'a state, psychic and sometimes also physical, characterised by a compulsion to take the drug on a continuous or periodic basis in order to experience its mental effects, and sometimes to avoid the discomfort of its abstinence (WHO 1974).

The DSM/IV definition of drug dependence describes "a maladaptive pattern of substance use leading to clinically significant impairment or distress associated with difficulty in controlling substance taking behaviour, withdrawal (signs and symptoms that occur when the drug is stopped and is alleviated by renewed administration of that drug, or one with similar actions) in the absence of the drug and tolerance (state in which drug actions diminish on repeated administration) to its effects".

Dependence can be categorised as physical and psychological. Psychological dependence is manifested by compulsive drug seeking behaviour in which the individual uses the drug repetitively for personal satisfaction,

despite risks to health. Physical dependence is defined in terms of the appearance of a physiological withdrawal syndrome.

The withdrawal syndrome, although clinically important, is not now believed to be important in the maintenance of dependence. The alcohol withdrawal syndrome manifests itself with overtly physical symptoms 6-8 hours after withdrawal. At this time point nausea, tremor and sweating are common. This stage is followed 48-72 hours later by a delirious and tremor stage, where the person undergoing withdrawal may suffer visual and auditory hallucinations, anxiety, restlessness, and paranoia, and in severe cases, epileptic seizures can occur (Victor & Adams, 1953). Neuronal hyperexcitability during alcohol withdrawal is generally treated with benzodiazepines, such as diazepam (Feuerlain & Reiser, 1986). Diarrhoea and tremor, caused by excessive sympathetic nervous system activity, can be treated with either $\beta 2$ adrenoceptor antagonists (Kraus et al., 1995) or $\alpha 2$ adrenoceptor agonists (Baumgarter et al., 1987). These treatments only offer relief from the symptoms of the withdrawal syndrome they do not treat the underlying mechanisms of alcohol dependence.

Most alcoholics including those abstaining, experience craving, often for extended periods of time. Focusing research on the long-term effects of alcohol may provide information about the causes of relapse back into drinking. A major influence on relapse is conditioning, returning to the environment where drug administration occurred being a powerful stimulus. A further characteristic of relapse is 'priming', where a small intake of the drug, even long after abstinence, results in a strong desire for more drug. Both of these phenomena suggest that neurochemical changes, as yet undefined, are still present long after the initial withdrawal signs subside.

Treatment

Clinically, pharmaceutical agents used in the treatment of alcoholism include disulfiram, the opiate antagonists naltrexone and nalmefene, serotonergic agents such as ondansetron, buspirone, and the selective serotonin reuptake inhibitors such as citalopram, fluoxetine, paroxetine and sertraline, and other agents including lithium and acamprosate (calcium acetyl homotaurinate) (Alcohol Alert 2000).

Disulfiram inhibits aldehyde dehydrogenase and leads to increased levels of acetaldehyde when alcohol is consumed, with subsequent adverse physical effects such as nausea, headache and weakness. There is little evidence that disulfiram enhances abstinence. The opiate antagonists, naltrexone and nalmefene, cause a hypothesised reduction in the rewarding properties of alcohol by blocking opioid receptors. There is good evidence that naltrexone reduces relapse and number of drinking days in alcohol-dependent subjects. There is some evidence that naltrexone reduces craving and enhances abstinence in alcohol-dependent subjects. (O'Malley et al., 1992; Volpicelli et al., 1992).

Acamprosate has been reported to reduce drinking days in alcohol-dependent subjects (Lhuintre et al., 1985, 1990). In experimental animal models acamprosate induces a significant reduction of alcohol consumption (Boismare et al., 1984; Le Magnen et al., 1987a & b); Gewiss et al., 1990; Littleton 1995). Some signs of physical withdrawal such as hyperactivity and hyper-reactivity are reduced by acamprosate (Gewiss et al., 1990; Spanagel et al., 1996). Initially it was proposed that acamprosate interacted with $GABA_A$ receptors however, it does not interact with $GABA_A$ binding sites nor does it influence $GABA_A$ stimulated chloride currents (Zieglgansberger et al., 1997). There is evidence for

interactions between acamprosate and NMDA receptors and also voltage activated calcium channels (Al-Qatari & Littleton, 1995, 1998). The exact mechanism of its action is as yet not fully elucidated.

The effects of serotonergic agents on alcohol consumption in animal models of alcohol consumption are described later in this chapter. In clinical studies there is some evidence on the efficacy of serotonergic agents for the treatment of alcohol-dependent symptoms in patients with comorbid mood or anxiety disorders, although the data is limited (Johnson, 2000). Clinical trials of serotonergic agents in primary alcoholics without comorbid mood or anxiety disorders have provided minimal evidence on the efficacy of serotonergic agents for treatment of the core symptoms of alcohol dependence (Johnson & Ait-Daoud, 2000).

Alcohol and the central nervous system

Alcohol appears to have widespread actions on the CNS, affecting a range of neurotransmitters, neuromodulators and ion channels. These include actions on GABA, glutamate, dopamine, serotonin, acetylcholine and various neuropeptides. Alcohol was originally believed to act via effects on membrane lipid fluidity (Overton, 1896). However, not all substances that affected membrane fluidity caused intoxication, and alterations in temperature that disrupted membrane fluidity did not produce effects similar to alcohol. The site of action has also been proposed to be a disruption of lipid protein interactions, and the membrane proteins themselves. None of the mentioned mechanisms is exclusive. There is strong evidence for a direct action of alcohol upon ion channels; results from patch clamp experiments on cell attached patch and inside-out patches have suggested that the site of action of alcohol on NMDA

receptors is located on a domain located on, or only accessible from, the extracellular environment (Peoples and Stewart, 2000). The alcohol-binding site is thought to consist of a hydrogen bond acceptor within the protein; binding at this site may alter the local protein structure leading to alcohol's effect (Dwyer & Bradley, 2000).

The mechanisms by which alcohol acts on the brain to modify behaviour are better understood through investigations on the action of alcohol on the functions of specific neurotransmitters. Studies of neurotransmitters and the receptors, which they bind, have provided data on both the mechanisms of action of alcohol on these molecules as well as clues to their roles in alcohol related behaviour. Extensive and comprehensive reviews on alcohol and neurotransmitter interactions are widespread in the literature. An exhaustive review of all the interactions between alcohol and all the neurotransmitters, neuromodulators, ion channels, and receptors would be beyond the scope of this thesis because of the vast array of alcohol's actions and reactions. The following accounts are a brief summary of the main, or important effects of alcohol on the major receptor systems. More detailed information is provided, where relevant, in subsequent chapters.

GABA

GABA is the main inhibitory neurotransmitter in the CNS. It acts through three main types of receptors the ionotrophic $GABA_A$ and $GABA_c$ receptors and also the metabotrophic $GABA_B$ receptor. Sedative and anxiolytic drugs such as the benzodiazepines and barbiturates exert their action by enhancing GABAergic transmission. The similarity of their actions to the sedative/anxiolytic effects of alcohol has led to much investigation on alcohol-GABA interactions.

Electrophysiological studies showed that high doses (100 mM) of alcohol had no effect on $GABA_B$ receptors (Frye & Fincher, 1996). However, the $GABA_B$ receptor has been implicated in the alcohol induced inhibition of NMDA responses (Steffensen, et al 2000).

$GABA_B$ receptor activation increased alcohol-induced anaesthesia and decreased alcohol preference (defined by a two bottle choice between water and dilute alcohol) (Daoust et al., 1992; Martz et al., 1983) Chronic alcohol induced excitability was unaffected by $GABA_B$ agonists in mice (Mead & Little, 1995; Molleman & Little, 1995). $GABA_B$ receptor activation decreased withdrawal related anxiety in rats (File et al, 1992).

Electrophysiological studies on alcohol and $GABA_A$ receptor have not shown consistent results. Alcohol enhancement of GABA mediated inhibition has been shown in the cerebellum of rats, but only in the presence of noradrenaline (Freund & Palmer, 1996). Alcohol induced increases in GABA mediated inhibition of neurons has been shown in the cerebral cortex (Nestoros, 1980), spinal cord (Celentano et al., 1988) and the hippocampus (Takada et al., 1989). Whereas other researchers have shown no effect of alcohol on $GABA_A$ receptor function (Carlen et al., 1982, Siggins et al., 1987). Chronic exposure to alcohol potentiates $GABA_A$ mediated responses in the rat periform cortex when measured during alcohol treatment (Signore & Yeh, 2000). $GABA_A$ receptor density has been shown to be increased when measured both during chronic alcohol treatment, and immediately on withdrawal after chronic alcohol treatment (Unwin & Taberner, 1980), but others report no change in electrophysiological change in receptor function after chronic alcohol when measured six or twenty-four hours into withdrawal (Buck & Harris 1990).

Hippocampal neurons showed no changes in any aspect of $GABA_A$ receptor-mediated transmission during alcohol withdrawal (Whittington et al., 1991a). The potency of alcohol to modulate the activity of $GABA_A$ receptors has been shown to be linked to cell type and subunit composition. This order of potency is: DRG neurons > $\alpha 1\ \beta 2\ \gamma 2s$ > cortical neurons (Mori et al, 2000)

Glutamate

Two main classes of glutamate receptor exist, ionotrophic (ligand gated ion channels) and metabotrophic (G-protein coupled receptors). There are two main subgroups of ionotrophic glutamate receptors; NMDA receptors and non-NMDA (AMPA /kainate) receptors. These receptors are distinguished by the relative selectivity of exogenous ligands (NMDA, AMPA and kainate) to active the receptors.

In rat cultured neurons, alcohol directly inhibited NMDA ion currents (Lovinger et al 1989). AMPA/kainate receptor currents induced by low levels of kainate are more effectively reduced by alcohol than those induced by high kainate levels (Dildy-Mayfield and Harris, 1992). Alcohol at concentrations of between 10 mM and 100 mM decreased NMDA-induced $Ca2^+$ influx (Leslie & Weaver, 1993; Hoffman et al., 1989) and reduced NMDA induced release of dopamine, noradrenaline and acetylcholine (Gothert and Fink 1989; Woodward and Gonzales 1990) amongst other neurotransmitters. It also reduced excitatory NMDA transmission in isolated neurons as well as in brain slices and in vivo (Leslie & Weaver, 1993). NMDA responses to alcohol vary according to location and subunit composition of the receptor (Crews et al., 1983).

Increases in glutamate transmission and increases in voltage activated calcium channels play an important role in neuronal hyperexcitability seen

during alcohol withdrawal. Chronic alcohol has been shown to increase NMDA receptor binding sites. Membrane and autoradiographic studies have shown significant increases in the binding densities for MK-801 (a NMDA receptor ligand), 4-8 hours after withdrawal from seven-day liquid diet treatment (Gulya et al., 1991, Guppy et al., 1995) and in cultured cerebellar granule cells following a 3-day incubation with 100 mM alcohol (Hoffman et al., 1985). Human alcoholic post mortem brain tissue has shown increased NMDA-receptor density (Freund & Ballinger, 1991). Chronic alcohol treatment has been shown to increase both AMPA/kainate and NMDA receptor mediated transmission in the hippocampus after withdrawal from chronic alcohol treatment (Shindou et al., 1994; Molleman & Little 1995a,b; Whittington et al., 1995). The changes in the activity of hippocampal pyramidal cells during alcohol withdrawal may reflect the behavioural signs of withdrawal (Whittington & Little, 1990, 1991, Whittington et al., 1991,1992, 1995).

Dopamine

Activation of the mesolimbic dopaminergic system by alcohol in rodents has been shown using a variety of methods. Increases in dopamine and its metabolites in the ventral striatum have been demonstrated using alcohol doses of 1-3g/kg (Barbaccia et al., 1982). The mesolimbic dopaminergic system has been implicated in reinforcement processes in general and enhanced mesolimbic dopaminergic transmission is a common property of most drugs of abuse (Di Charia & Imperato 1985, Samson et al., 1992).

The alcohol-induced stimulation of dopamine release in the nucleus accumbens is dependent on endogenous opioid peptides. Opioid antagonists prevent alcohol stimulated dopamine release. Acute alcohol administration can

increase the firing rate of dopaminergic neurons of the VTA (Brodie et al., 1988). Chronic administration of alcohol decreased dopamine outflow from the nucleus accumbens, with a decline in mesolimbic dopaminergic neuronal activity that persists as long as 72 hours after withdrawal (Diana et al., 1996). Studies involving in vivo electrophysiological recordings in un-anaesthetised animals have also demonstrated reduced firing rates and altered firing patterns of neurones in the VTA following withdrawal from chronic alcohol treatment. (Diana et al., 1995; Grant et al., 1994). Additional information on the alcohol and dopamine interactions is discussed in Chapter 7.

Noradrenaline

The primary areas for the origin of noradenergic neurons in the CNS are the pons and the medulla. These nuclei consist of the following main groupings; the locus coerulus complex and its caudate extension, the lateral tegmental cell system, and the dorsal medullary system (Lindvall & Bjorkland, 1974).

Experiments investigating the effect of alcohol on noradrenaline have not been conclusive. Administration of FLA-57, a dopamine beta-hydroxylase inhibitor resulted in a reduction of alcohol consumption in rats (Amit et al., 1970). However, lesion studies report an opposite effect, with alcohol consumption increasing after noradrenergic lesions and the acquisition of oral self-administration of alcohol has been abolished by destruction of the dorsal noradrenergic bundle in rats (Kianmaa et al., 1995). Alpha$_2$ adrenoceptor antagonists have been shown to reverse the ataxic and hypothermic effects of acute alcohol administration in mice (Durcan et al., 1991, 1992) and acute i.p. injections of 2g/kg alcohol have been reported to stimulate the synthesis and turnover of noradrenaline (Lyness & Smith, 1992)

5HT

Serotonin (or 5HT) is a neurotransmitter widely distributed in the CNS. Up to seven 5HT receptor subtypes have been identified ($5HT_1$, $5HT_2$...$5HT_7$). The $5HT_1$, $5HT_2$, $5HT_3$ and $5HT_4$ receptors have been pharmacologically well characterised. $5HT_1$ and $5HT_2$ receptors can be subdivided into $5HT_{1A}$, $5HT_{1B/D}$, $5HT_{1E}$, $5HT_{1F}$ and $5HT_{2A}$, $5HT_{2B}$ and $5HT_{2C}$ respectively. $5HT_3$ receptors are ligand gated ion channels whereas the other receptor subtypes are G-protein coupled receptors. The cell bodies of 5HT neurons are mainly located in the medial and dorsal raphe nuclei. 5HT fibres project to the forebrain and terminate in several cortical areas, as well as in other brain structures such as the striatum, the nucleus accumbens, the ventral tegmental area, the amygdala and the hippocampus (Jacobs and Azmitia, 1992).

There is evidence that drugs of abuse including alcohol may influence brain 5HT activity and that 5HT may have a role in the regulation of specific aspects of addictive behaviour. Electrophysiological, neurochemical and behavioural studies suggest that the effects of alcohol in the CNS are mediated in part though action at the $5HT_3$ receptor complex. Alcohol at doses of between 25mM and 100mM enhances cation conductance at the $5HT_3$ receptor (Lovinger, 1991; Lovinger & White, 1991). $5HT_3$ receptor antagonists block the discriminative stimulus effects of alcohol and block the ability of alcohol to release dopamine from the nucleus accumbens, and reduce alcohol intake measured by preference procedures (McBride et al., 1991).

An alcohol induced decrease in $5HT_{1C}$ and $5HT_{2A}$ receptor activity has been demonstrated in xenopus oocytes at 50-150mM (Sanna et al., 1994). In hippocampal neurons alcohol potentiates 5HT1A and $5HT_4$ receptors (Lau &

Frye, 1996). Alcohol consumption in both humans and experimental animals is reduced by drugs that increase 5HT synthesis and/or release, including $5HT_{1A}$, $5HT_{1B}$, and $5HT_{1C}$ agonists and 5HT reuptake blockers (Murphy et al, 1988; McBride et al 1991). Therapeutically, in humans drugs, which activate serotonergic transmission, appear to be of some potential value in reducing alcohol consumption although as yet serotonergic drugs are of limited value clinically (Johnson, 2000).

Levels of 5HT and the metabolite 5-hydroxyindoleacetic acid (5HIAA) has been found to be lower in the hippocampus, nucleus accumbens, striatum, cortex and the hypothalamus of different alcohol preferring, compared with alcohol non-preferring, rats (Zhou, 1994). Research has indicated that $5HT_3$ receptor antagonists can reduce alcohol drinking in alcohol preferring rats but the efficacy of these drugs is reduced with chronic administration. (Rodd-Hendricks et al., 2000)

Acetylcholine

Acetylcholine acts at two main classes of receptors, muscarinic and nicotinic acetylcholine receptors. At concentrations of alcohol above 100mM the effect of applied nicotine was potentiated on receptors expressed in Xenopus oocytes (Covernton & Connelly, 1997) although blood alcohol levels of 100mM have been reported to be fatal to humans (Charness et al., 1989). Electrically stimulated acetylcholine release has been shown to be inhibited by alcohol possibly through the actions of other neurotransmitters (Hoffman & Tabakoff, 1985). Microdialysis has produced data that shows a biphasic effect of alcohol on acetylcholine release in the hippocampus, with 20mM stimulating release and higher concentrations having an inhibitory effect (Henn et al 1998). The acute

actions of alcohol on mesolimbic dopamine release are blocked by the nicotinic antagonist, mecamylamine (Blomquist et al., 1992). Effects of prolonged alcohol administration on nicotinic receptors have been reported (Nordberg et al., 1985). Central nicotinic acetylcholine receptors in the VTA are involved in modulating the mesolimbic dopaminergic pathway activating effects of alcohol (Soderpalm 2000). Administration of quaternary autonomic drugs have indicated that enhanced alcohol intake may involve ganglionic and or peripheral muscarinic transmission (Ericson et al 2000).

The effects of chronic alcohol treatment on central cholinergic systems have been well studied. In human alcoholics the number of cholinergic neurons is reduced, as well as reduced activity of the acetylcholine-synthesizing enzyme, choline acetyltransferase (Arendt et al., 1983) this has also been reported in animal studies (Arendt et al., 1988). Reductions in muscarinic receptor density have been shown in the frontal cortex, hippocampus and putamen of human alcoholics (Freund & Ballinger, 1991). Long-term alcohol administration appears to reduce cholinergic transmission.

Opioids

There is evidence that alcohol induced activation of the endogenous opioid system may be part of a neurobiological mechanism that is involved in mediating alcohol drinking behaviour. Acute alcohol administration increases endorphin (Naber et al 1981, Barret et al 1987) and enkephalin gene expression (Schulz et al 1980). Administration of μ and δ opioid antagonists reduces both alcohol preference and self-administration of alcohol in animal models (Froechlich et al 1991). The opioid antagonist naltrexone is used in the treatment of alcohol dependence in humans and has efficacy in preventing relapse in

alcoholics (O'Malley et al 1992, Volipicelli et al 1992). Antagonists at the μ and δ receptors block alcohol induced dopamine release from striatal brain slices (Widdowson & Holman 1992). Chronic alcohol administration has been shown to up-regulate δ receptor binding in the rodent brain measured immediately on withdrawal from a 21-day liquid diet treatment (Gianoulakis, 1986; Charness et al., 1983).

Voltage dependent ion channels

Voltage dependent sodium channels are relatively unaffected by behaviourally relevant alcohol concentrations. High doses of acute alcohol inhibit sodium influx, IC50 of 500mM (five times the fatal blood alcohol concentration). The inhibition of sodium flux is an action shared with general anaesthetics and closely related to membrane-disordering effects and the effects of alcohol (10 – 100 mM) on sodium channels are particularly important in mediating the behavioural effects of alcohol (Harris et al., 1987).

Potassium influx is increased in cerebella Purkinje neurons by 20 mM alcohol (Carlen et al, 1982), the locus ceorleus (60 mM) and in the rat hippocampus alcohol facilitates potassium flux via the calcium activated potassium channel at concentrations above 20 mM (Carlen et al., 1982, Yound & Sigman, 1981).

Voltage activated calcium channels are affected by behaviourally relevant concentrations of alcohol, the effects are specific to channel types and to brain region. An alcohol induced decrease in calcium influx has been reported in N, P/Q, and L type channels (Wang et al 1991ab) as well asT type channels (Twombly et al 1990). Dihydropyridine-sensitive calcium channels have previously been shown to be involved in alcohol dependence. Prolonged intake

of alcohol results in an increased number of dihydropyridine-sensitive binding sites in the central nervous system (Dolin et al., 1987).

Administration of dihydropyridine calcium channel antagonists decreased the development of tolerance to alcohol (Dolin and Little, 1989), decreased the appearance of behavioural and electrophysiological signs of withdrawal (Whittington & Little, 1991; Whittington et al., 1991) and prevented the up-regulation of the dihydropyridine binding sites (Dolin and Little, 1989; Whittington et al., 1991). Cortical membranes showed an increase in binding that rises sharply after 3-4 days of alcohol consumption and is maintained until about 24 h after cessation (Guppy et al, 1995). The mechanism of this up-regulation is as yet unresolved. The increase in binding site densities may result from several processes, including the presence of a different membrane environment, posttranslational modification of the protein, or the uncovering of cryptic sites, or may be a result of de novo synthesis (Guppy et al, 1995). Investigations of the mechanism of this up-regulation in cultured cells showed that the up-regulation was prohibited by addition of the mRNA synthesis inhibitor, anisomycin (Brennan & Littleton, 1990).

Alcohol doses and behaviour

Acute alcohol administration has been shown to affect a variety of behavioural processes. In humans, low blood alcohol concentrations (5 mM) impair motor co-ordination and elevate mood (Charness et al 1989). Increased concentrations (>10 mM) are associated with increased road traffic accidents (Verbanck, 1995). Concentrations of (20 mM) produce ataxia and sedation (Charness et al 1989). Loss of consciousness is reported at high doses (50 mM) and death at (100 mM). Generally higher blood alcohol concentrations are required in rodents for

analogous behavioural effects, in mice the loss of righting reflex is seen at blood concentrations of 65mM (Charness et al 1989).

A study measuring the blood alcohol concentrations of mice after administration of alcohol by liquid diet (as used in some of the following studies) have shown with a mean daily alcohol consumption of 20g/kg/day, that produces blood alcohol concentrations that are in excess of 20 mM (Jones 93 unpublished data). Peak blood alcohol concentrations of 105 mM during liquid diet administration of alcohol have been reported (Shih et al., 1996).

Stress and alcohol

The term "stress" often is used to describe the subjective feeling of pressure or tension or to the physiological processes initiated in response to a stressor. The stress response is a complex process; the association between drinking and stress is more complicated still. Multiple genetic and environmental factors determine addictive behaviour and the individual response to stress. Studying the link between alcohol consumption and stress may further our understanding of drinking behaviour.

The stress response

The maintenance of the body's relatively steady internal state, or homeostasis, is essential for survival. Biochemical and physiological function is challenged by a wide variety of stressors - illness, injury, and psychological factors (depression, fear, etc.). Stressors prompt physiological and behavioural changes in a process of continual adaptation, with the goal of maintaining homeostasis and coping with the stress. Selye defined stress as "the non-specific response of the body to any demand" (Selye, 1976). The non-specific response

can arise from a plethora of stimuli some noxious and life threatening (attack and injury) to more benign stimuli such as a novel environment. Both the perception of what is stressful and the physiological response to stress vary considerably among individuals. These differences are based on genetic factors and environmental influences that can be traced back to infancy. The responses of an individual to a stressor may also vary depending upon the specific situation (De Kloet et al 1997). Different stressors may activate different neurochemical pathways and elicit the release of ACTH and corticoids (cortisol & corticosterone) through different neuroendocrinological mechanisms (Dallman et al 1987).

The stress response is an integrated network involving the central nervous system, the adrenal system, and the cardiovascular system. The hypothalamus at the base of the brain, initiates the stress response by secreting corticotrophin releasing factor (CRF). CRF triggers a series of physiological and behavioural reactions, it stimulates the secretion of adrenocorticotrophin hormone (ACTH) from the pituitary gland (also known as corticotrophin) into the circulation. The adrenal glands, located at the top of the kidneys, in turn are stimulated to secrete glucocorticoid hormones. The main glucocorticoid hormones secreted in humans is cortisol (in rodents it is corticosterone). Termination of the stress response is controlled by negative feedback. Corticosterone inhibits the secretion of ACTH and CRF; ACTH inhibits the secretion of CRF. (See Figure 1.1, which shows the HPA axis and feed back loops). Activation of the stress response affects smooth muscle, fat, the gastrointestinal tract, the kidneys, and many other organs and the body functions that they control. The stress response affects the body's regulation of temperature; appetite and satiety; arousal, vigilance, and attention; mood; and

more. Physical adaptation to stress allows the body to redirect oxygen and nutrients to the stressed body site, where they are needed most.

Figure 1.1 The Hypothalamic adrenal pituitary axis

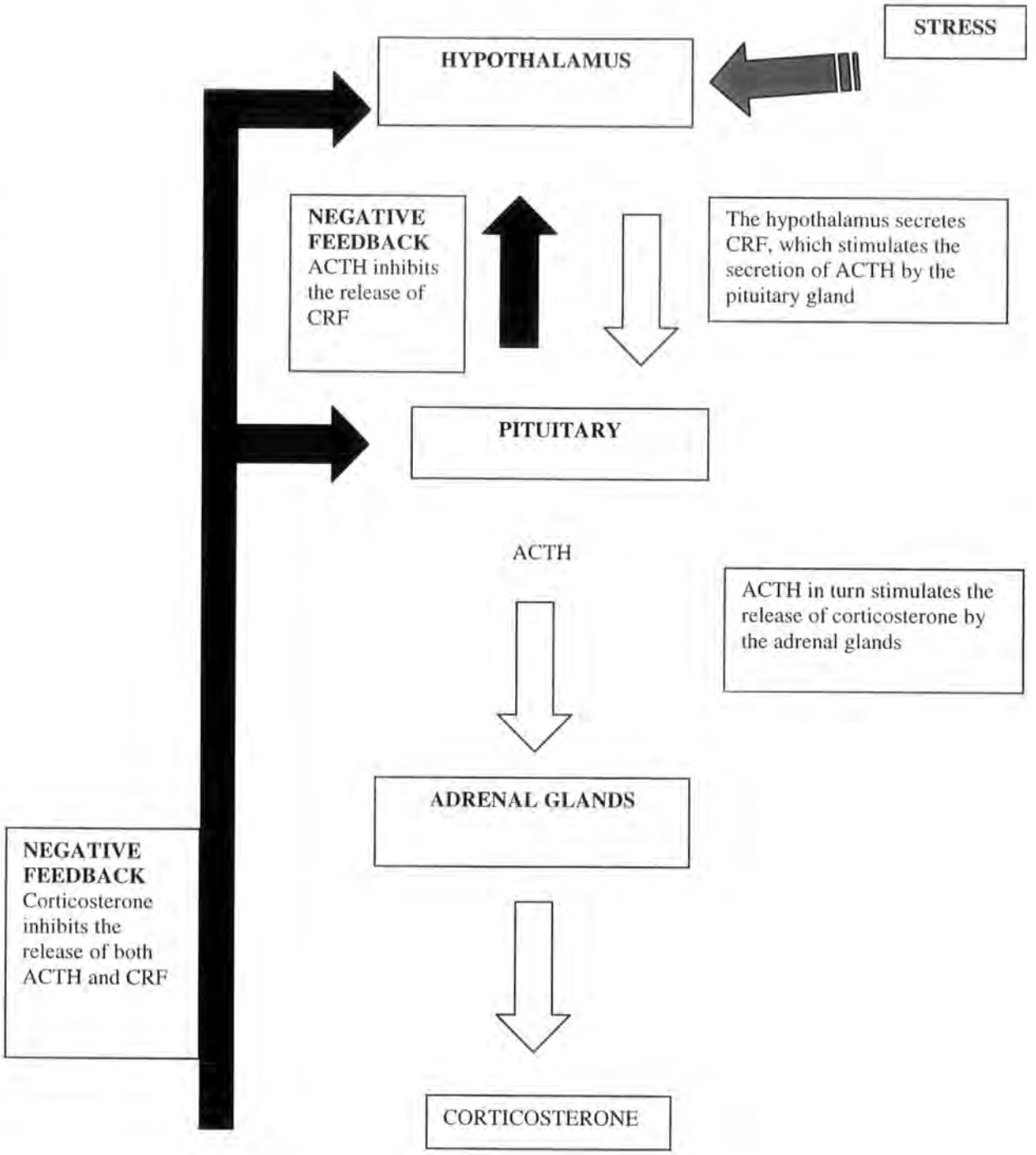


Figure 1.1 *The hypothalamus at the base of the brain, initiates the stress response by secreting corticotrophin releasing factor (CRF). CRF triggers a series of physiological and behavioural reactions, it stimulates the secretion of adrenocorticotrophin hormone (ACTH) from the pituitary gland (also known as corticotrophin) into the circulation. The adrenal glands, located at the top of the kidneys, in turn are stimulated to secrete glucocorticoid hormones. Termination of the stress response is controlled by negative feedback. Corticosterone inhibits the secretion of ACTH and CRF; ACTH inhibits the secretion of CRF.*

Stress is usually thought of as harmful; but when the stress response is acute and transient, homeostasis is maintained and few adverse effects result. Under chronic stress, however, when the body either fails to compensate or when it overcompensates, damage can occur. Such damage may include suppression of growth, immune system dysfunction, and cell damage can result in impaired learning and memory.

There is considerable recent experimental evidence that stress plays a major role in the development of dependence on drugs of abuse, but the mechanisms involved are not yet fully understood. The majority of experimental studies that have demonstrated links between stress and dependence have been carried out on drugs that are psychostimulants (Piazza & Le Moal, 1996), but corresponding evidence is beginning to emerge for alcohol, as described below. Some effects of stress on CNS function have been identified, but it has been recognised only recently that stressful experiences may have long-term effects on the function of the neuronal pathways that are thought to be involved in the development of dependence (Piazza et al., 1993). Stress hormones can cause functional changes in many of the neuronal systems on which alcohol acts, but the mechanisms involved in the effects of stress on alcohol consumption require elucidation.

The importance of stress in the acute situation as a contributing factor in alcohol consumption was suggested many years ago, in the "tension-reduction" hypothesis (Pohorecky, 1981). This stated that the acute anxiolytic actions of alcohol are a major cause of alcohol consumption, because people drink alcohol in order to obtain the acute effect of relief of anxiety. This is likely to be an

important factor contributing to the consumption of alcohol, particularly in social situations, and the resultant high consumption in individual cases may increase the likelihood of subsequent development of dependence. There is also evidence that alcohol itself stimulates the stress response. Alcohol administration has been shown to increase the production of adrenocorticotrophin (ACTH) and increase plasma corticosterone concentrations (Ellis 1966). There is now evidence that more long term changes may occur as a result of stressful experiences, involving alterations in CNS function that last beyond the acute subjective experience of stress (Post, 1992; Bulwalda et al., 1999) for example. Lasting behavioural changes in rats after a single exposure to a cat (Adamec, 1994). Footshock stress induced long lasting behavioural changes together with persistent alterations in the hypothalamic-pituitary-adrenal (HPA) axis (Desan et al., 1988)

In clinical studies, it has been found that a higher incidence of stressful major life events is associated with alcohol dependence (Gorman & Brown, 1992). Employment in occupations that provided high strain and low control was found to be associated with an increased risk of alcohol abuse (Crum et al., 1995). Chronic stressful experience that was personally threatening was found to be associated with an increased risk of relapse drinking in abstinent alcoholics (Brown et al., 1990). The mechanism of these effects is uncertain, although prolonged changes in HPA function are found in abstinent alcoholics. A decreased ACTH response to administered corticotrophin releasing factor (CRF) has been frequently reported in abstinent alcoholics (Adinoff 1990; Ehrenreich et al., 1997) and also blunted ACTH and cortisol release in response to stress (Vescovi et al., 1997), although the latter may recover more quickly during the abstinence period than the former (Ehrenreich et al., 1997). Increased serum

cortisol concentrations have also been reported in alcoholics (Farren et al., 1995) and decreases in cortisol binding globulin in the plasma have also been observed (Hiramatsu and Nisula, 1989; Frajria and Angeli, 1977).

The role of stress in alcohol consumption has been the subject of recent experimental studies. Early work demonstrated increases in voluntary alcohol drinking after stress produced by immobilisation (Rockman et al., 1987; Nash and Maikel, 1985) or footshock (Volpicelli et al., 1986), although not all studies have found such an effect with these stressors (Ng Cheong Ton et al. 1983; Fidler and Lolordo, 1996). A consistent pattern has been the demonstration of increased alcohol consumption after these more severe stressors in low preference animals, with less, or no, effect on the preference of individuals with high preference prior to the stress. This pattern was seen by Volpicelli et al. (1986) and Rockman et al. (1987).

Much experimental evidence now implicates the hormones of the HPA axis in dependence on drugs of abuse. A possible mechanism for the involvement of corticosterone in drug abuse can be seen in Figure 1.2. Rats will self-administer corticosterone to achieve plasma concentrations of this hormone, 1-1.5 mM, that are in the stress range (Piazza et al., 1993) and will also self-administer ACTH (Johaneau-Bowers & Le Magnen, 1979). Adrenalectomy reduced self-administration of cocaine by rodents, while the acquisition of such self-administration was increased by corticosterone (Goeders & Guerin, 1996). Prevention of corticosterone synthesis by administration of metyrapone reduced the relapse rate of rats previously self-administering cocaine (Piazza et al., 1994). Corresponding behavioural evidence regarding corticosterone has also been obtained for alcohol consumption. Administration of corticosterone increased

alcohol consumption in rats and administration of metyrapone or adrenalectomy decreased alcohol intake (Fahlke et al., 1994a; 1994b; 1995). The situation for the other stress hormones is less clear; both ACTH and CRF have been found to decrease voluntary consumption of alcohol by rodents (Krishnan et al., 1991; Bell et al., 1998).

Figure 1.2

Corticosterone and reward

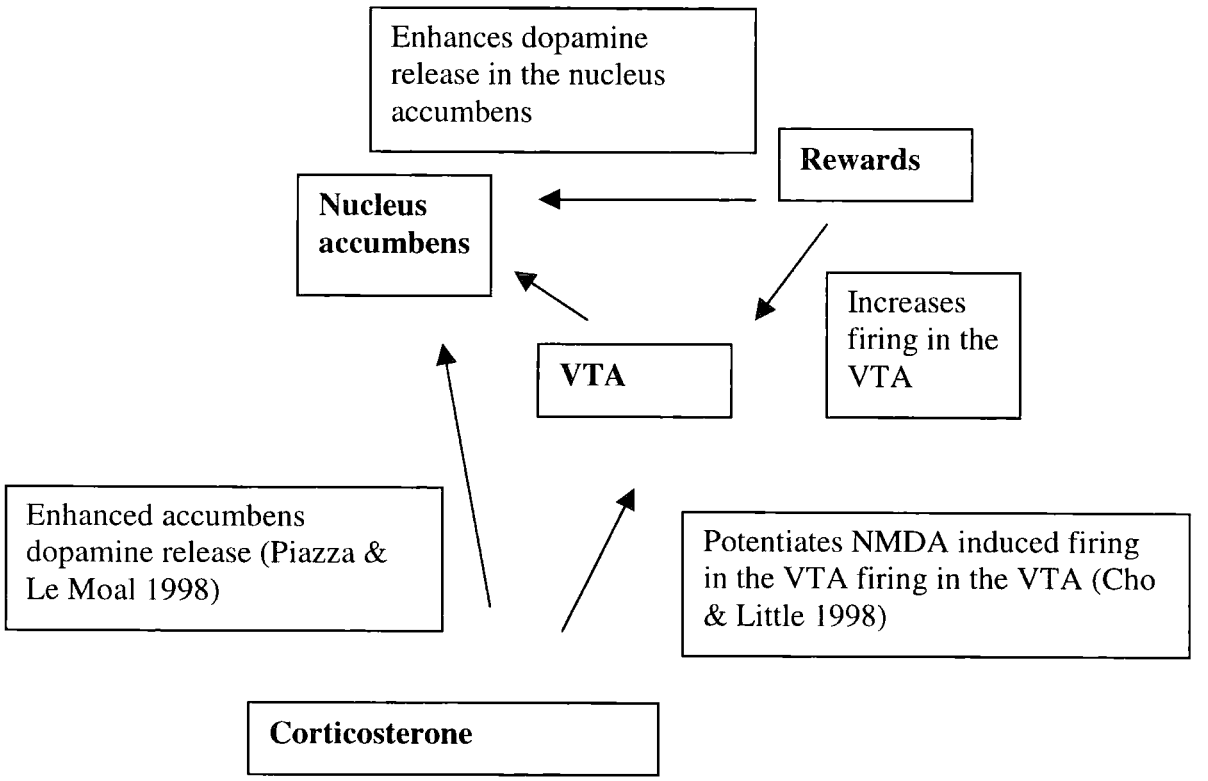


Figure 1.2 *Possible interactions between corticosterone and the mesolimbic dopaminergic system. Corticosterone may activate or enhance the 'reward system' either by increasing VTA firing or enhancing accumbal dopamine release.*

Animal models of dependence

There is no ideal model for alcohol dependence in animals; an ideal model of alcohol dependence in animals should mirror human alcohol dependence, involving oral consumption of alcohol in sufficient quantities to produce dependence. Alcohol should be consumed in free choice preference to other fluids irrespective of taste, smell or calorific value resulting in relevant blood alcohol concentrations. Alcohol should be consumed in sufficient quantities to cause tolerance and physical dependence. That is expression of the symptoms of alcohol withdrawal syndrome upon cessation of treatment.

Various methods have been developed to produce alcohol intake as alcohol has been reported to have an aversive taste to rodents. All researchers do not however universally hold this reported aversion to the taste of alcohol. Reid (1996) reports that " contrary to laboratory folklore, most rats will consume alcoholic beverages and some will become zealous consumers" he does qualify this statement with " rats do not, however, avidly consume alcohol under all circumstances". The favourite circumstance appears to be when alcohol is presented as a sweetened saccharin solution (0.25%); this does not really produce a strong argument against the aversive taste of alcohol.

Administration to experimental animals has been performed by a variety of methods; as sole drinking fluid (Mello, 1973), via repeated injections (e.g. Freud, 1969), by replacing water and food with an alcohol containing liquid diet (e.g. Lieber & Decarli, 1986), and inhalation of alcohol vapour (e.g. Goldstein & Pal., 1971). Despite none of these methods reaching the ideal model for specific experimental situations each have their own advantages.

Administration of dilute alcohol as sole drinking fluid has been used to produce physical dependence in rodents (Mello, 73). High alcohol preferring strains of rodents; Fawn Hooded, P & HAD rats (Li et al., 1993, 1994: Li and McBride, 1995) and C57 (Unwin & Taberner, 1982) mice have all ingested consistently high doses of alcohol when presented as sole fluid. Chronic treatment with 24% alcohol as sole fluid has been shown to lead to the production of physical dependence in C57 strain mice (Whittingham & Little, 1988).

Alcohol can be injected intra-gastrically (i.g) or intra-peritoneally (i.p) (Yanagita et al 1969) this method has disadvantages as with the rapid metabolism of alcohol it is difficult to attain consistent and stable blood alcohol levels and frequent injections become necessary. Also, repeated injections of alcohol have been reported to damage rat intestines at doses of 20% alcohol v/v. Inhalation of alcohol produces consistent high blood alcohol levels however alcohol intake in this method is not voluntary and inhalation rather than ingestion is also less than ideal. Laboratory animals selectively bred for high and low alcohol consumption constitute a widely accepted animals model of experimental alcoholism.

It has been reported that if a high alcohol-drinking animal is to be used to model for screening therapeutic agents and to elucidate the biochemical basis of alcoholism, then the animal should meet certain criteria. The animal should drink extraordinary amounts of alcohol, for comparison to other models the pattern of the amounts of alcohol drunk and the preference for alcohol over water should be comparable to other lines. In addition the desire to consume alcohol should remain in the presence of highly palatable but necessarily rewarding solutions. Different drugs previously shown to decrease alcohol consumption by either

alcoholic or other animal models should reduce the consumption of alcohol in the new animal model. (McMillen & Williams, 1995). The consumption of alcohol in the presence of another highly palatable liquid (flavoured chocolate drinks are the normal substance used) is not necessarily a good constraint on the model of alcohol consumption in any given rodent strain, as the palatable distracter may itself be 'rewarding'. There is consistent evidence linking the consumption of sweets and alcohol in both humans and animals (Kampov-Polevoy et al., 2000).

Prolonged neuronal changes

High rates of relapse to drug use after long periods of abstinence characterise the behaviour of experienced users of drugs of abuse (Jaffe, 1990), 66.5% of alcoholics who had undergone a period of abstinence had once again become alcoholics after 3 years of initial treatment (Helzer, 1985).

There is both behavioural and neurochemical evidence that chronic intake of alcohol can have very prolonged effects. Recent work has shown that the effects of amphetamine and cocaine on locomotor activity of mice were increased up to two months after cessation of chronic administration of alcohol by liquid diet (Manley and Little, 1997), which supports the possibility of long term neurochemical changes. Electrophysiological evidence has been obtained showing prolonged changes in VTA neuronal activity after the cessation of withdrawal hyperexcitability following chronic alcohol treatment (Bailey & Little, 1997; Bailey et al., 1998).

Aims

The high probability of relapse amongst abstaining alcoholics presents an important area for research. Understanding the neuronal mechanisms that cause relapse may help uncover more effective treatments for alcohol dependence. One

of the factors that are reported to be important in relapse is stress. The mechanisms that underlie the relationship between stress and alcohol consumption and relapse to consumption are poorly understood. The experiments performed in this thesis were designed to provide more information on the relationship between stress and alcohol consumption.

Activation of the HPA axis resulting in increased levels of corticosterone, CRF and ACTH is believed to play a role in both alcohol consumption and relapse to alcohol drinking. The aim of this thesis is to investigate the influence of corticosterone, CRF, ACTH and stressful environmental manipulations on alcohol consumption. This thesis also aims to investigate potential long-term neurochemical changes both in the mesolimbic dopaminergic system and the HPA axis that may provide information to explain the neurochemical basis of relapse.

Section one

Alcohol preference studies

This section (chapters two, three, four and five) reports on preference studies performed on C57 mice. Originally bred by C.A. Little in 1921, the C57 strain of mouse has been extensively used in alcohol research because of its reported high alcohol preference (McLearn and Rodgers, 1959; Phillips and Crabbe 1991; Belknap et al., 1993). The animals used in the following set of experiments are C57/BL10 (line ScSn) from the stock originally bred in Bristol Medical School. The breeding line was subsequently moved to Durham University.

Although, the C57/BL10 mouse has been widely used as an "alcohol preferring" strain i.e. this strain fits the criteria described in the introduction, these mice, in our laboratory, demonstrate considerable variability in alcohol consumption. (Watson et al., 1996). When placed in a free choice situation between a bottle of dilute (8%) alcohol and a bottle of water, a considerable percentage demonstrate a low preference for alcohol, consuming less 30% of their total fluid as water (an alcohol preference ratio of less than or equal to 0.3); others demonstrate a high preference for the dilute alcohol, drinking over 70% of their total fluid as dilute alcohol (an alcohol preference ratio of greater than or equal to 0.7) (Little et al., 1999). The alcohol preference distribution is seen in both sexes. Selective breeding from the low preferring and high preferring mice produces a similar preference distribution as that from unselected breeding pairs (Little et al., 1999). Similar variability in preference is also seen in mice of the

C57/BL6 strain (Little et al., 1999), so the variation in alcohol consumption is not confined to the line bred in our laboratories.

The majority of neurochemical studies on C57 mice and alcohol preference have used the BL6 substrain. Ng et al. (1996) demonstrated lower enkephalin gene expression in mesolimbic areas in alcohol-naive C57/BL6J animals, compared with the DBA/2J strain, which was increased by alcohol consumption.

With the C57 we have an inbred (genetically identical) strain of mouse that demonstrates two distinct behavioural phenotypes i.e. high and low alcohol preference. Vulnerability to alcohol dependence has its roots (at least in part) in a variety of genetic and early environmental factors, to age, stress and co-existing psychoactive conditions. C57 mice have a genetic vulnerability to high alcohol consumption, yet some of these mice do not consume high levels of alcohol. Uncovering the neuronal factors behind this could be an important step in understanding the basis of individual vulnerability to alcohol dependence.

The use of selectively bred alcohol preferring strains of rodent is a valuable tool in alcohol research. High alcohol preferring animal models have been used, with some success, in testing pharmacotherapies for alcoholism. Various agents that alter 5HT, dopamine, GABA and opiate functioning have been shown to decrease alcohol consumption in high alcohol preferring rodent lines (Lumeng et al., 1995). The opioid antagonist naltrexone, in addition to reducing alcohol consumption in high alcohol preferring rodent strains, has been shown to decrease the mean number of drinking days per week, the frequency of relapse, and reduce the desire to drink (O'Malley et al., 1992; Volipicelli et al., 1992). Reductions in human, and selective rodent strain alcohol drinking by the

same agents supports the predicative validity of the use of selectively bred strains for evaluating therapies. It also supports their use in the basic scientific research into the behaviour and neurochemistry of alcohol use and dependence.

Chapter Two

Predicting Alcohol preference

Introduction

Background

To the majority of people, alcohol drinking is an enjoyable social pastime, but to some it can present a dependence problem. The individual variation in vulnerability to the reinforcing effects of addictive drugs is a clinical feature of the development of dependence. Large individual differences in conditioned and unconditioned responses to drugs have been noted in experimental animals (Piazza & Le Moal 1996). Although it is widely accepted that genetic and environmental influences underline the basis of individual vulnerability to alcohol abuse or dependence, the exact mechanisms for this are poorly understood. The ability to predict alcohol preference or susceptibility to drug self-administration would provide a powerful tool in uncovering vulnerability to drug a breeding of laboratory rodents for alcoh nd alcohol dependence.

Selective breeding

The aversion most rodents have of alcohol has led to the selective ol preference, in order to develop a rodent model for alcoholism. A number of selectively bred lines of high and low preferring rats have been bred including; Alcohol-Preferring (P) and Alcohol-Nonperferring (NP), High Alcohol Drinking (HAD) and Low Alcohol Drinking (LAD). High alcohol consumption has also

been discovered in strains that were originally bred for other purposes for example the Fawn Hooded strain demonstrates high alcohol consumption but was originally bred for research into a blood disorder arising from 5HT transporter dysfunction in platelet cells (Kirchmaier et al., 1990).

Selected lines of high alcohol preferring rats naturally show similar high alcohol drinking but, given the myriad of factors influencing home cage drinking and the selective breeding procedure for their creation, they undoubtedly represent different genotypes. Neurochemical, and immunohistochemical comparisons between high and low preferring rats have demonstrated a number of consistent differences between high and low preferring lines. Amongst the neurotransmitter systems notably implicated in high alcohol consumption is 5HT (e.g. Murphy et al., 1982, Gongwer et al., 1989, Wong et al., 1988; Zhou et al., 1994). One of the most consistent neurochemical and neuroanatomical findings seen in P/NP and HAD/LAD rats is the deficiency of 5HT in the alcohol preferring lines (McBride et al., 1991). Compared with rats that drink small amounts of alcohol, the high alcohol-consuming rats have reduced 5HT in several brain regions including the prefrontal cortex and the nucleus accumbens. These differences may be caused by a relative reduction in 5HT containing axons in these areas and a compensatory up-regulation of 5HT receptor activity (Li & McBride 1995). High preferring rat lines have also shown a higher density of GABAergic axon terminals in the nucleus accumbens. (McBride et al., 1991). The endogenous opioid systems are involved in the regulation of alcohol drinking. High and low alcohol-drinking lines differ in their opioid activity in the absence of alcohol as well as in alcohol-stimulated opioid activity in the nucleus

accumbens and pituitary gland (Nylander et al., 1994). In the present study the high alcohol preferring C57 strain of mice, a 'high' preferring strain was used.

Methods of predicting alcohol preference

Reports have shown that saccharin-induced increases in daily fluid intake in selectively bred rat lines (Knapp et al., 1997), and saccharin intakes in heterogeneous rat lines (Overstreet et al 1993) can predict ethanol consumption. However, the relationship between voluntary saccharin intake and alcohol intake has been brought into question recently, as it has not consistently demonstrated (Overstreet, 1999). Other measurements, including immobility in a forced swim test, and time spent on the open arms of the elevated plus maze, have been reported to correlate positively to alcohol consumption. However, these measures are not consistently associated with high alcohol intake (Knapp et al., 1997).

The observation by Prasad and Prasad (1995) of an association between high basal levels of corticosterone and an enhanced corticosterone response to stress and high voluntary alcohol consumption in rats is of particular interest. This proposed link between corticosterone and alcohol consumption fits with other observation on corticosterone's influence on alcohol consumption (Fahkle & Hansen 1999). Chronic stress is known to cause a variety of behavioural manifestations some of which include an enhanced individual predisposition to drug-seeking behaviour (Piazza and Le Moal, 1996). The mechanism by which high basal corticosterone levels influence alcohol consumption is not understood. The observed link between corticosterone and alcohol consumption may be mediated by the effect of corticosterone on one or more of these neurotransmitters and neuromodulators that are also involved in alcohol (and

other drugs of abuse) intake. It is also possible that the circulating levels of corticosterone may directly influence alcohol consumption.

Locomotor activity and drug self-administration

The reaction of animals to a novel environment, activation of the HPA and exploratory behaviour, provide the basis of a method that is reported to be able to predict susceptibility to drug self-administration. Piazza and co-workers have shown that naive rats when exposed to a novel environment, exhibit a wide range in locomotor activity; this observed locomotor activity is positively correlated to psychostimulant self-administration (Piazza & Le Moal 1996). Psychostimulants, as well as opiates, nicotine and alcohol exert their initial effects through different sites in the brain. They all cause similar changes in the mesolimbic dopamine pathway, acutely enhancing transmission in the mesolimbic dopaminergic system from the VTA to the nucleus accumbens. The enhanced dopaminergic activity in the nucleus accumbens has been postulated to be the primary factor in determining the susceptibility to high levels of drug self-administration (Koob and Bloom 1988; Piazza & Le Moal 1998).

Studies using comparisons between high responding and low responding (for operant self-administration) rats selected as such on the basis of their locomotor activity in a novel environment have demonstrated a number of neurochemical differences between the groups of rats. High responders were shown to have reduced expression of tyrosine hydroxylase and cholecystokinin (CCK) in the mesencephalon and increased expression of preproenkephalin, preprodynorphin and preprotackykinin in the striatum and nucleus accumbens (Lucas et al., 1996).

In the many substrains of C57 mice in addition to the ethanol preference other common patterns of behaviour have been described. In general C57 mice show high levels of open field exploration, with low levels of anxiety-related behaviour in an open field, and high learning capacity in many memory tasks (Crawley et al., 1997). The BL/10 substrain has a high startle reactivity (Paylor and Crawley, 1997). However, no clear correlations have been found between ethanol preference and particular behaviours or the effects of alcohol on behaviour (Phillips and Crabbe, 1991).

Aims

The aims of this study were (i) to characterise the previous observations on consumption and preference, (ii) to measure blood corticosterone concentrations, prior to screening for alcohol preference, and to examine the relationship between alcohol consumption and corticosterone levels to examine whether corticosterone levels could be used to predict preference. (iii) And to discover whether spontaneous locomotor activity could predict subsequent alcohol preference.

Methods

Stock animals

All the mice were "bred in house". After weaning (19 to 21 days) the mice were transferred to new cages in single sex groups of 10 per cage. The cages contained mice from different litters. They were housed at 21 ± 1 °C, with 55 ± 10% relative humidity, and a 12 hour light/dark cycle, with the light phase between 08:00h and 20:00h, and free access to tap water and laboratory rodent chow (CRM) at all times.

Alcohol preference measurements

Mice were taken from the stock cages and individually housed with two fluid bottles made continuously available to them. One bottle containing tap water and the other, alcohol (100% ethanol, Fisher Scientific) diluted with tap water to a concentration of 8% v/v. Measurements of fluid intake were made, three times per week (Monday, Wednesday and Friday, at 09.00 - 10.00 a.m.). The amount drunk from each bottle was used to calculate the ratio between 8% ethanol and water. The screening was continued for 3 weeks, as the mice show a stable alcohol preference by the third week of screening. The mean of the 3 measurements in the third week of screening was used to assign the animals alcohol preference.

Experiment One

Characterisation of alcohol preference

This experiment was performed to investigate the relationship between alcohol preference and the amount of alcohol consumed (g/kg/day). Male mice, 25-35g (n=96), were screened for alcohol preference using the standard screening procedure described below.

Experiment Two

Prior locomotor activity and preference

This experiment was performed to investigate whether alcohol preference could be correlated with locomotor activity. Male C57 mice (25-30g), n=46, were taken from their stock cages, between 10 a.m. and 4 p.m. (during the light phase), and then placed into locomotor activity meters and spontaneous activity was monitored for 30 minutes. These mice were then screened for alcohol preference using the alcohol preference screening protocol described earlier. A control group, n=46, was screened for alcohol preference in parallel, but without exposure to the activity meters. Therefore accounting for the effects of the stress of the procedure. Locomotor activity and preference screening were performed in the same animal holding room.

Locomotor activity measurement

Spontaneous locomotor activity prior to screening was measured using Opta-Varimex-Mini activity meters operated by the interruption of 15 infra-red beams. A clear perspex cage (50 x 32 x 15 cm), containing a small amount of sawdust was placed between a metal frame containing the infra-red emitters and

sensors placed 1 inch apart. The mice were placed in the locomotor activity boxes for 30 minutes prior to undergoing the alcohol preference procedure. Static, mobile, and rearing activities were monitored.

General (or total) locomotor activity does not refer to a single class of behaviour; depending on the recording technique, it can comprise many different motor movements. For this reason the activity measurements were divided into static, mobile and rearing counts. Static activity was measured by counts of beam breaks in a non-sequential fashion, e.g. grooming and digging in the sawdust. Mobile activity was measured by the consecutive breaking of beams. Rearing activity was counted when the upper layer of beams was broken.

Experiment Three

Corticosterone levels and preference

The purpose of this experiment was to investigate whether there was any correlation between total plasma corticosterone levels and alcohol preference. Immediately prior to the screening procedure, blood samples were taken from two groups of stock male C57 mice (25-45g). One group of mice (n=23) had blood samples taken between 8 and 9 am (at the start of the light phase - *lights on*) and an other group (n=23) between 8 and 9 p.m. (at the start of the dark phase - *lights off).* Parallel control groups were used; these underwent the same handling procedure but did not have a blood sample taken. The samples were then used to assay the total corticosterone levels, using anti-corticosterone antibodies supplied by Sigma (see later). After the mice had their blood samples taken they were moved to another holding room for alcohol preference screening as described earlier in experiment one.

A second experiment using the same protocol was performed on male C57 mice (25-30g, n=23 per treatment). The radioimmunoassay in this group was performed with antibodies supplied by ICN (see later). After these mice had the blood samples taken they were placed in single cages for screening, the screening procedure was performed in the same room as the blood samples were taken.

Blood sample collection

Blood sample collection was performed according to the method of Durschlag et al. 1996. The mouse was removed from its home cage. With the tail held in the middle with the thumb and forefinger, the middle or the ring finger stabilised the tail dorsal to the surface of the incision, while the mouse was restrained by slightly pressing on its back with the remaining fingers. Blood samples were obtained by lacerating the ventral blood vessels with the pointed end of a razor blade with a short, rapid motion slightly oblique to the vessels. The blood was collected in heparinized microtubes, and centrifuged at 5000xg 5°C for 15 minutes (Microcentaur, Sanyo). The plasma samples were then stored at -20°C until required for radioimmunoassay (RIA). After collecting the sample, the bleeding was halted by applying slight pressure to the wound with cotton buds.

Radioimmunoassay

Two suppliers of antibodies were used Sigma and ICN. The Sigma antibodies lost activity when stored (although they were stored in accordance with the manufacturer's instructions).

In radioimmunoassay, a limited amount of specific antibody is reacted with corresponding labelled tracer, in this case [^{3}H] 1,2,6,7, corticosterone (specific activity 65-70m Ci/mol Amersham, U.K.). Upon addition of increasing amounts of corticosterone, a correspondingly decreasing fraction of the tracer is bound to the antibody. After separation of the bound from the free tracer by dextran-coated charcoal, the amount of remaining radioactivity is counted and used to construct a standard curve against which the unknown samples are measured (see Figures 2.i and 2.ii).

Buffers used

Antibody diluting buffer:

0.05M Tris HCl pH 8,

0.1M NaCl,

0.1% NaN_3

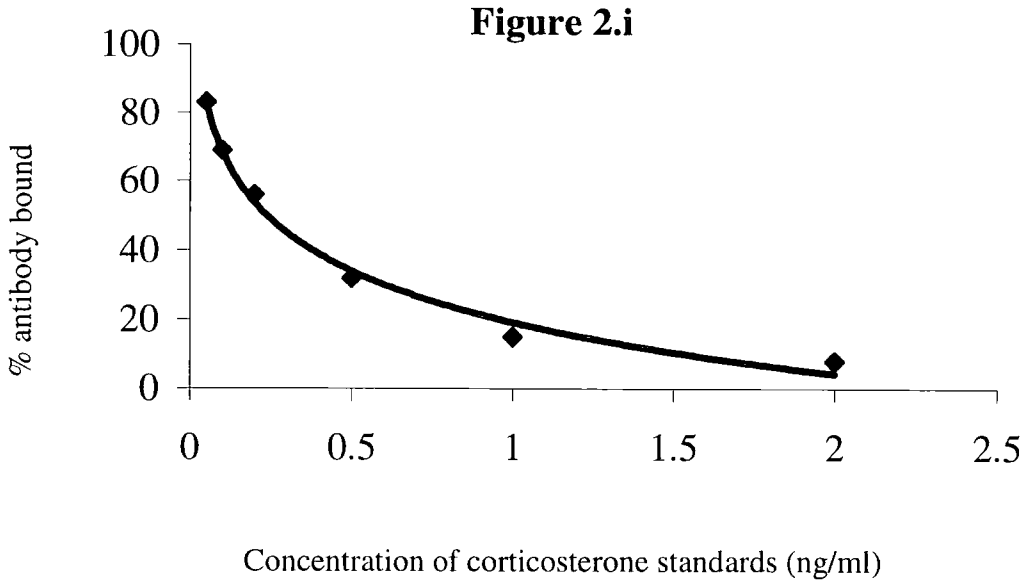
Tracer, sample, and charcoal slurry buffer:

0.05M Tris HCl pH 8,

0.1M NaCl,

0.1% NaN_3,

0.01% BSA



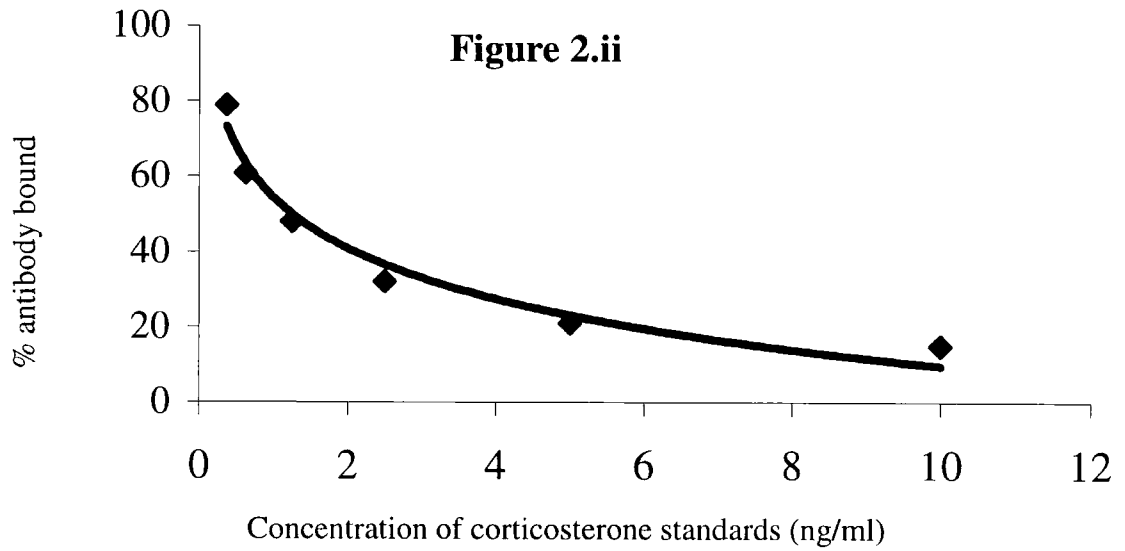


Figure 2.i and 2.ii *These figures show the standard curves generated for the corticosterone RIA measurements.* **Figure 2.i** *Shows the standard curve generated by the ICN-supplied antibodies and* **Figure 2.ii** *Shows the curve generated by the Sigma-supplied antibodies. Both types of antibody are specific of corticosterone showing <0.1% binding for other related steroids. The concentrations of the standards required by the two assays were different; the ICN-supplied antibody required dilution of the samples.*

Sigma supplied antibodies

A 0.1ml (diluted 1:10) volume of plasma or standard was pipetted into a 1.5ml eppindoff tube to which 0.5ml of antibody solution was added. The tubes were vortexed and allowed to stand for 30 minutes. The 0.1ml of 150pM tritiated corticosterone (in the modified Tris buffer previously described, with the addition of 0.1% BSA) was added and the tubes were incubated at 37°C for one hour. The tubes were then placed in an ice bath and when cooled 0.2 ml of 0.5% w/v dextran coated charcoal was added to remove unbound antibody. The tubes were then incubated for a further 15 minutes before being centrifuged at 4°C, x 2750g (bench top centrifuge) for 15 minutes. A portion of the supernatant was removed (0.25ml) to a scintillation vial and 3ml of scintillation fluid added, and the samples were then counted in a liquid scintillation counter (Tri Cab 2100 TR., Packard, Berks.).

ICN supplied antibodies

Plasma samples were diluted 1:5000 in antibody diluting buffer and 0.5ml aliquots were then heated to 98°C in a water bath to degrade the plasma proteins. The samples were allowed to cool and then 0.1ml of diluted labelled corticosterone (10,000 cpm) and 0.1ml of antibody were added. The tubes were mixed and then incubated at 4°C overnight. The following day 0.5ml of ice-cold 0.5% dextran-coated charcoal was added and the tubes were then incubated for a further twenty minutes on ice; finally the tubes were centrifuged (2750 xg) for 15 minutes at 4°C. The supernatant was decanted and 4ml of liquid scintillation fluid added before scintillation counting.

Statistical analysis

The correlation to alcohol preference from the corticosterone assays and locomotor activity measurements were analysed statistically using regression analysis. Comparisons of alcohol preference between treated groups were made using a Mann-Whitney test, for non-parametric data. Two-way analysis of variance, and where appropriate, Fisher's post-hoc tests, were used to analyse the RIA data.

Results

Experiment One

The mice demonstrated the biphasic distribution previously reported (Figure 2.1). The majority of mice were either high preferring (40%) or low preferring (44%) with few intermediate-preferring animals (16%). There was no significant difference in the total fluid consumption of high and low preferring mice (Figure 2.2). There was a positive correlation between alcohol preference ratio and g/kg/day of alcohol consumed ($y=12.65+0.068$; $r=0.94$, $t=26.719$, $P<0.0001$)(Figure 2.3).

Alcohol preference ratio did not correlate with total fluid consumption (Figure 2.4). Alcohol consumption in high preferring mice was up to ten times greater in g/kg/day (13 ± 0.5 g/kg/day) of alcohol than low preferring mice (2 ±0.2g/kg/day)(Figure 2.5).

All the data presented is the mean preference of the final three measurements of the three week screening. Previous work has shown that preference in these mice is stable after three weeks of screening (Little et al., 1999).

Figure 2.1

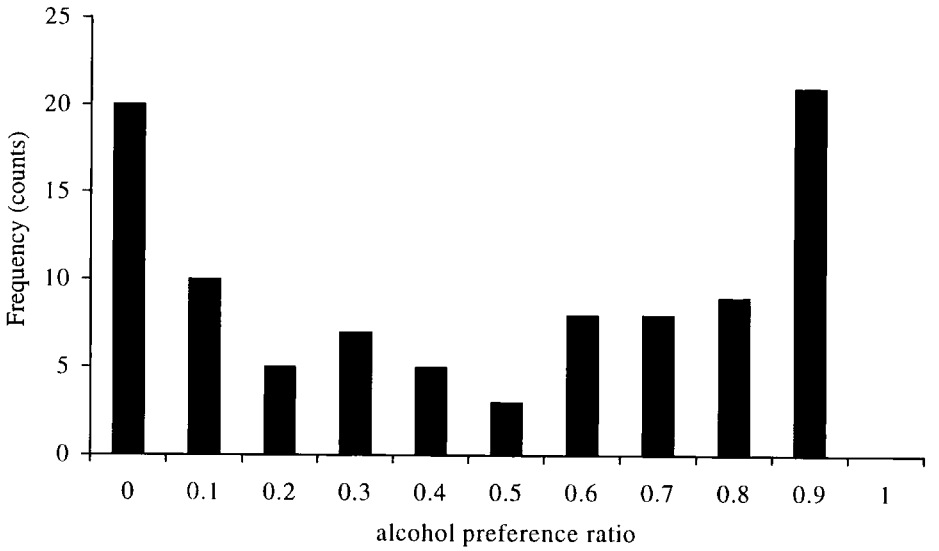


Figure 2.1 *The alcohol preference frequency distribution of male C57 mice (n-96) that underwent three week, two bottle, alcohol preference screening. The alcohol preference ratio is the ratio of the amount of dilute alcohol (8%) consumed divided by the total fluid intake.*

Figure 2.2

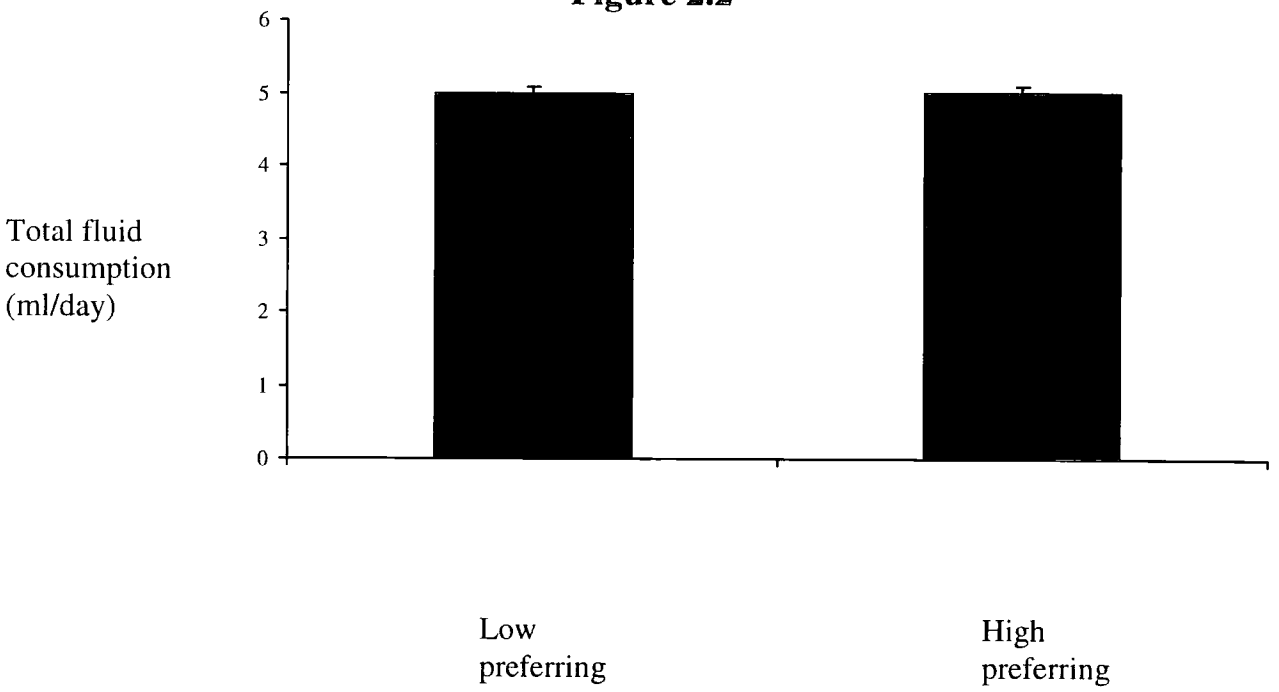


Figure2.2 *The mean total (+/-SEM) fluid consumption in high (n=35) and low preferring (n=41) male C57 mice after three weeks of preference screening.*

Figure 2.3

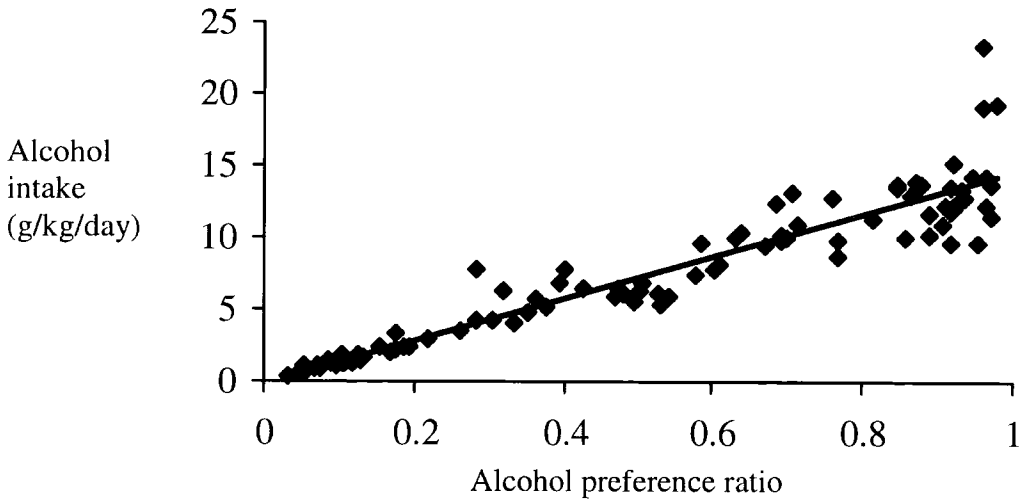


Figure 2.3 *The intake of alcohol (g/kg/day) and the alcohol preference ratio of C57 mice during alcohol preference screening. Correlation between alcohol preference and alcohol consumption in C57 mice. (r=0.94)*

Figure 2.4

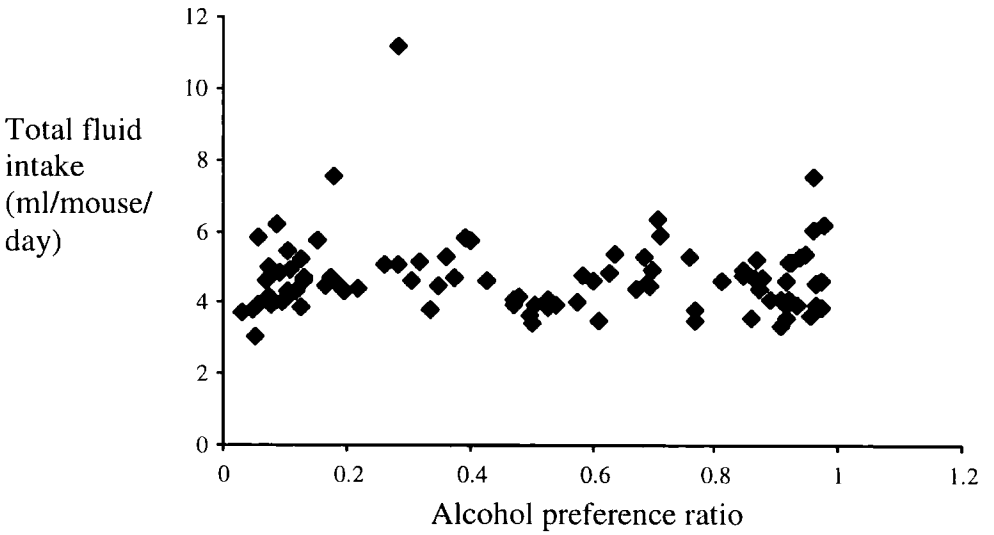


Figure 2.4. *The total fluid intake of male C57 mice during alcohol preference screening. Total fluid intake had no relationship to alcohol preference.*

Figure 2.5

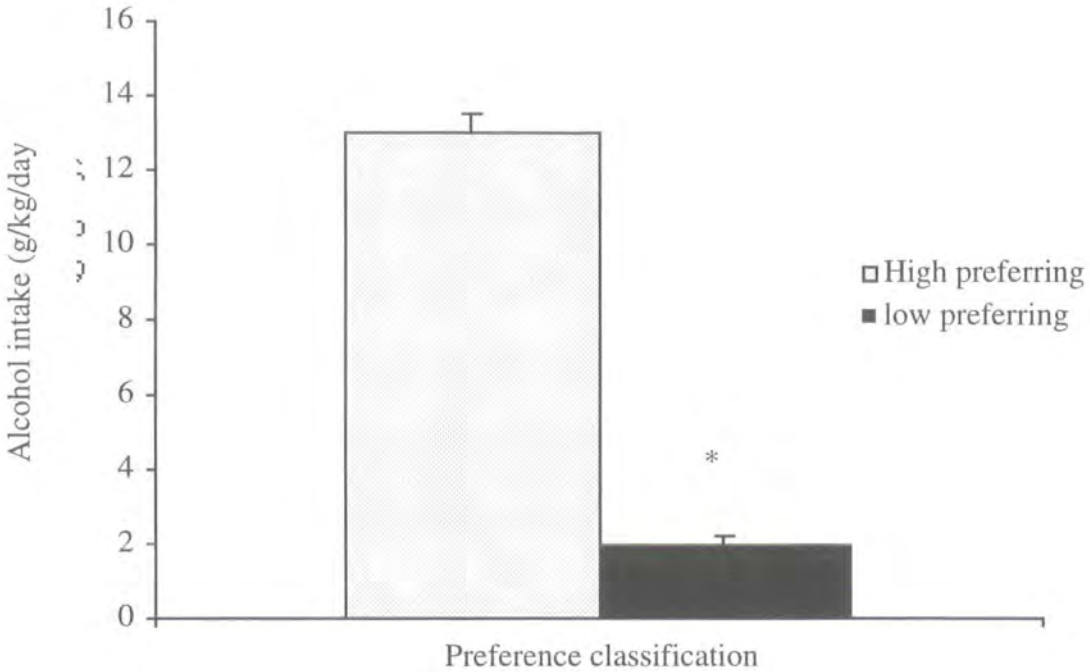


Figure 2.5. *The alcohol intakes in high (n=35) and low preferring (n=41) C57mice. High alcohol preferring mice consumed significantly more alcohol (g/kg/day) than low alcohol preferring mice. (*P<0.001).*

Experiment Two

Mobile locomotor counts between 0-15 minutes (Figure 2.6) and 16-30 minutes (Figure 2.7) showed no correlation with alcohol preference ($r<0.1$). Static locomotor counts between 0-15 minutes (Figure 2.8) and 16-30 minutes (Figure 2.9) showed no correlation with alcohol preference($r<0.1$). Rearing locomotor counts between 0-15 minutes (Figure 2.10) and 16-30 minutes (Figure 2.11) showed no correlation with alcohol preference ($r<0.1$). Alcohol preference was not dependent upon prior exposure to the activity meters. There was no significant difference between the preference ratios of the mice that were placed in the activity meters compared to those of mice that were screened without exposure to the activity meters ($P>0.5$).

Figure 2.6

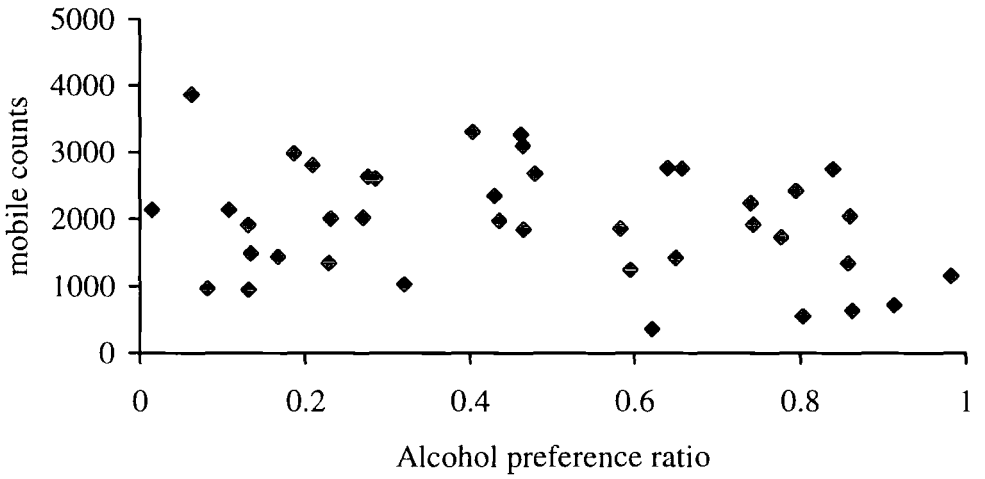


Figure 2.6 *Prior mobile locomotor activity and subsequent alcohol preference in C57 mice. There was no correlation between initial mobile locomotor activity (0-15 minutes) and alcohol preference after three weeks of alcohol preference screening.*

Figure 2.7

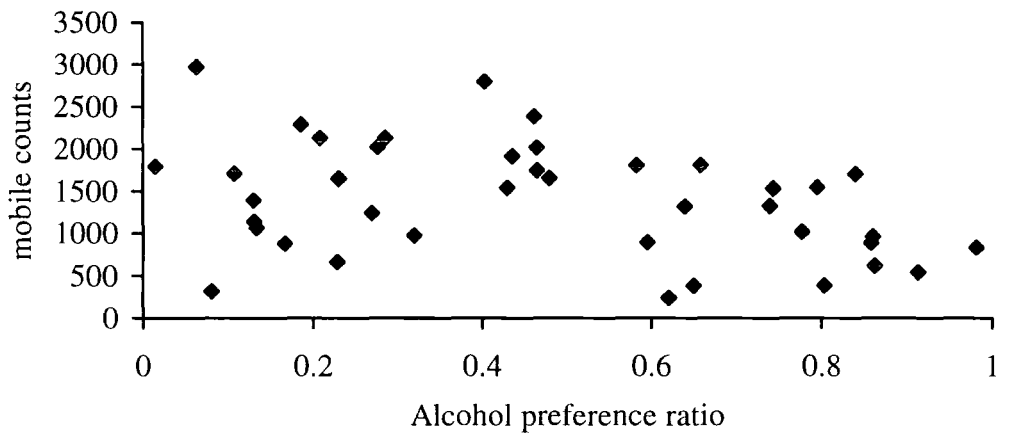


Figure 2.7 *Prior mobile locomotor activity and subsequent alcohol preference in C57 mice. There was no correlation between mobile locomotor activity (16-30*

minutes) and alcohol preference after three weeks of alcohol preference screening.

Figure 2.8

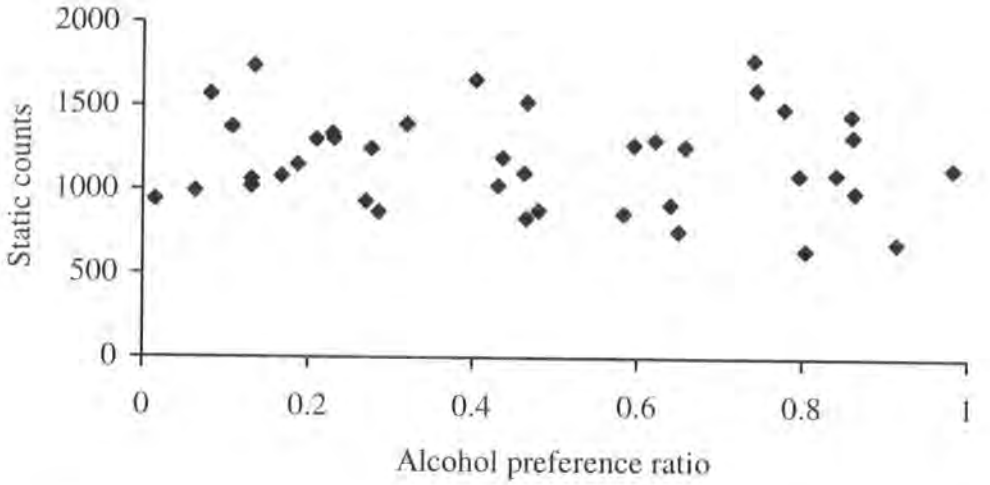


Figure 2.8 *Prior static locomotor activity and subsequent alcohol preference in C57 mice. There was no correlation between static locomotor activity (0-15 minutes) and alcohol preference after three weeks of alcohol preference screening.*

Figure 2.9

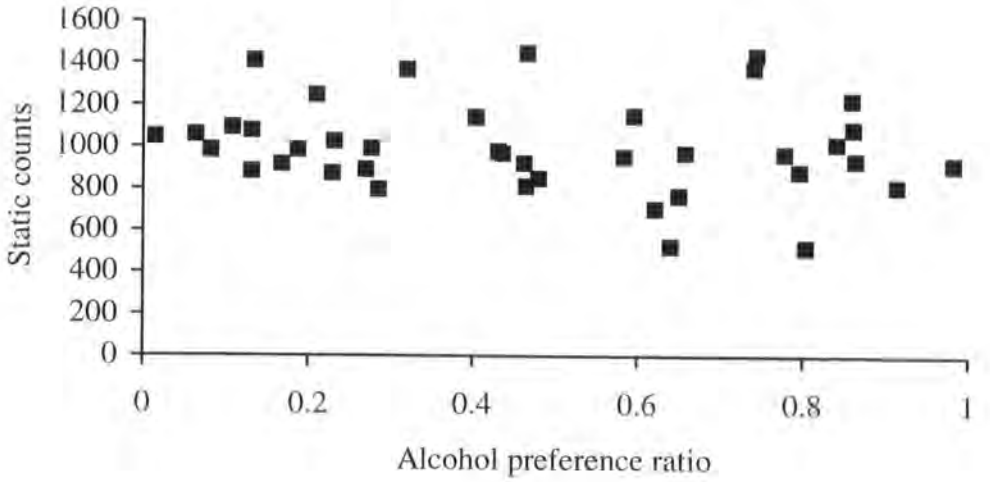


Figure 2.9 *Prior static locomotor activity and subsequent alcohol preference in C57 mice. There was no correlation between static locomotor activity (16-30 minutes) and alcohol preference after three weeks of alcohol preference screening.*

Figure 2.10

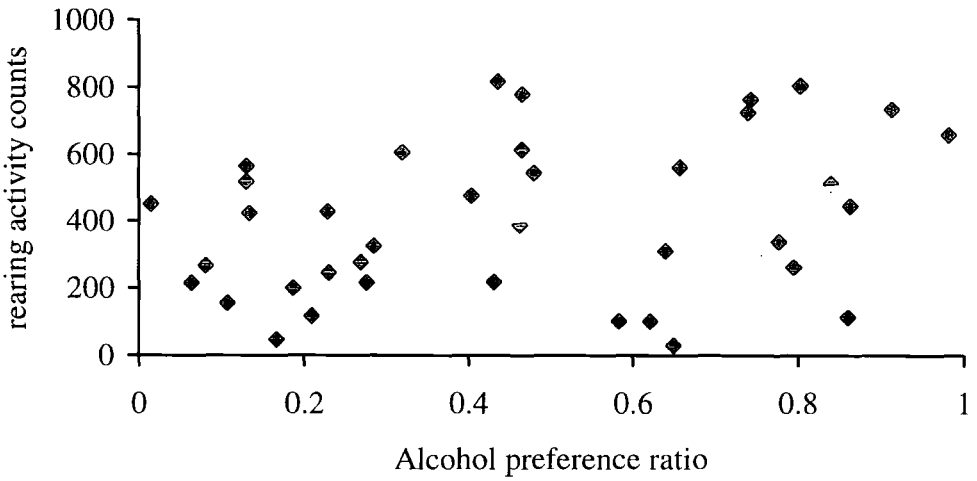


Figure 2.10 *Prior rearing locomotor activity and subsequent alcohol preference in C57 mice. There was no correlation between rearing locomotor activity (0-15 minutes) and alcohol preference after three weeks of alcohol preference screening.*

Figure 2.11

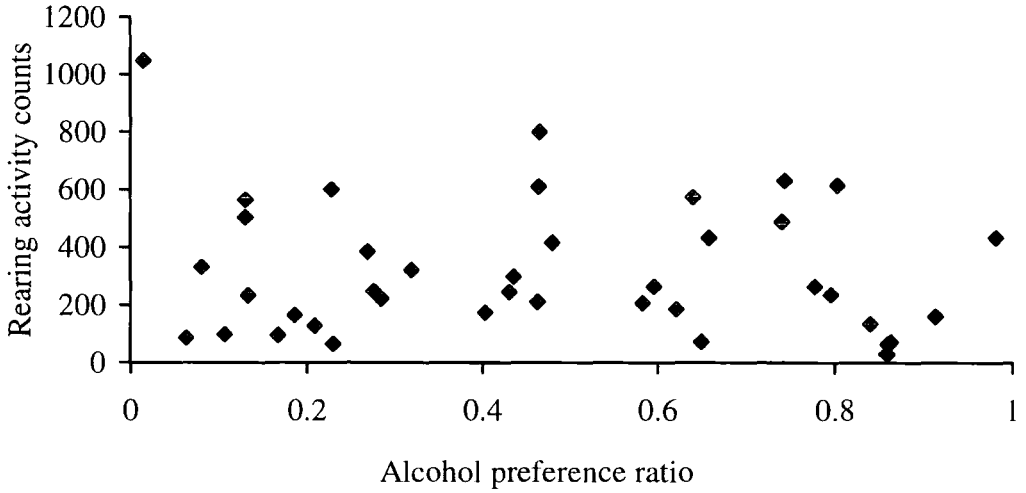


Figure 2.11 *Prior rearing locomotor activity and subsequent alcohol preference in C57 mice. There was no correlation between rearing locomotor activity (16-30 minutes) and alcohol preference after three weeks of alcohol preference screening.*

Experiment Three

Circadian fluctuations of corticosterone levels were seen in both the sigma and ICN assays. The corticosterone levels of high and low preferring mice at the respective time points did not differ, but the time at which the samples were taken affected corticosterone levels.

Higher levels of total corticosterone were seen at lights off using the sigma-supplied antibodies (lights on vs. light off $P<0.005$). The same pattern was seen in the assays that used the ICN supplied antibodies (lights on vs. light off $P<0.01$).

Corticosterone levels measured using the Sigma antibodies differed from levels assayed with ICN antibodies. The Sigma antibodies gave higher levels at both lights off and lights on compared to the ICN antibodies, ($F_{3,\ 65} = 18.53$ $P<0.0001$) (Figure 2.13c).

At either time point (in both methods), lights on or lights off, there was no correlation between corticosterone levels and alcohol preference, There was no relationship between corticosterone levels and alcohol preference in selected high, intermediate or low preferring populations (Figures 2.14 a, b, c and d).

There was no difference in the subsequent preference of mice that were either sampled or un-sampled (Fig 2.15). Also the time of day that the screening procedure was started (lights on or lights off) did not affect alcohol preference (fig 2.16).

Figure 2.13a
Corticosterone levels in C57 mice.
Sigma antibodies

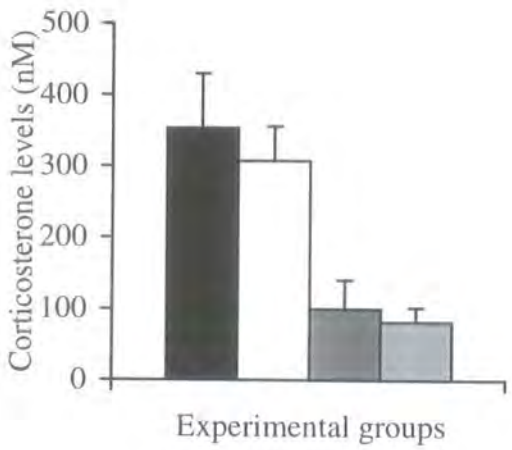
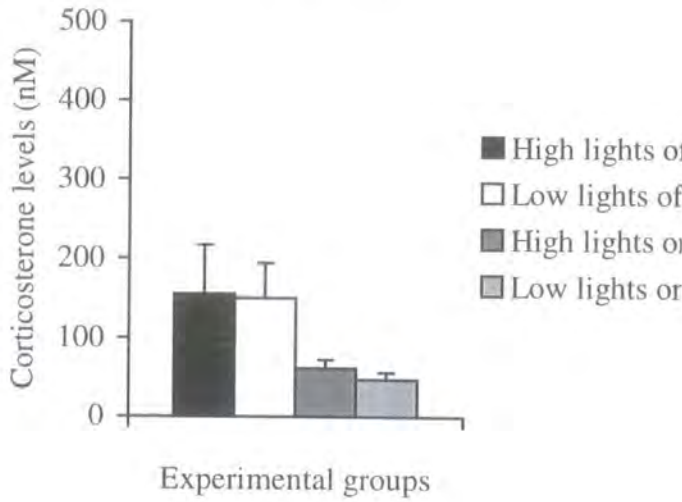


Figure 2.13b
Corticosterone levels in C57 mice
ICN antibodies



2.13c
Corticosterone levels at lights on and lights off using different antibodies

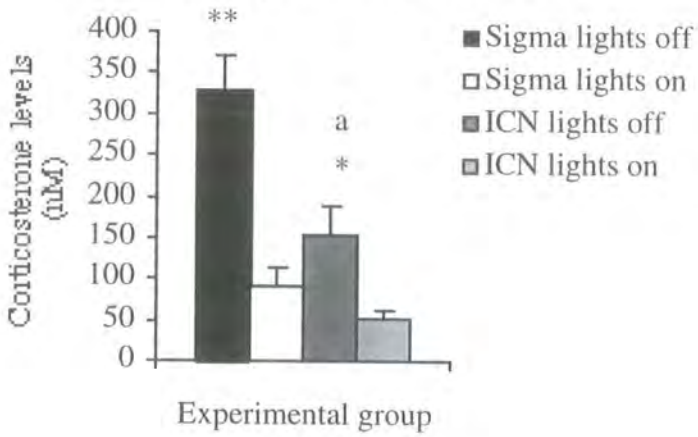


FIGURE 2.13 *Figure 2.13a Shows the corticosterone levels in high and low preferring mice measured using Sigma antibodies (n=9 per group); 2.13b Shows the levels using ICN antibodies (n=9 per group). Figure 2.13c show the levels measured by both methods in all the mice. (*p<0.01 ICN lights off vs. lights on; **P<0.001 Sigma lights on vs. lights off; two way ANOVA (supplier X time), $^{a}F_{3,65} = 18.5$ P<0.0001). Lights on describes the samples taken at the start of the light phase. Lights off describes the samples taken at the start of the dark phase.*

Figure 2.14a

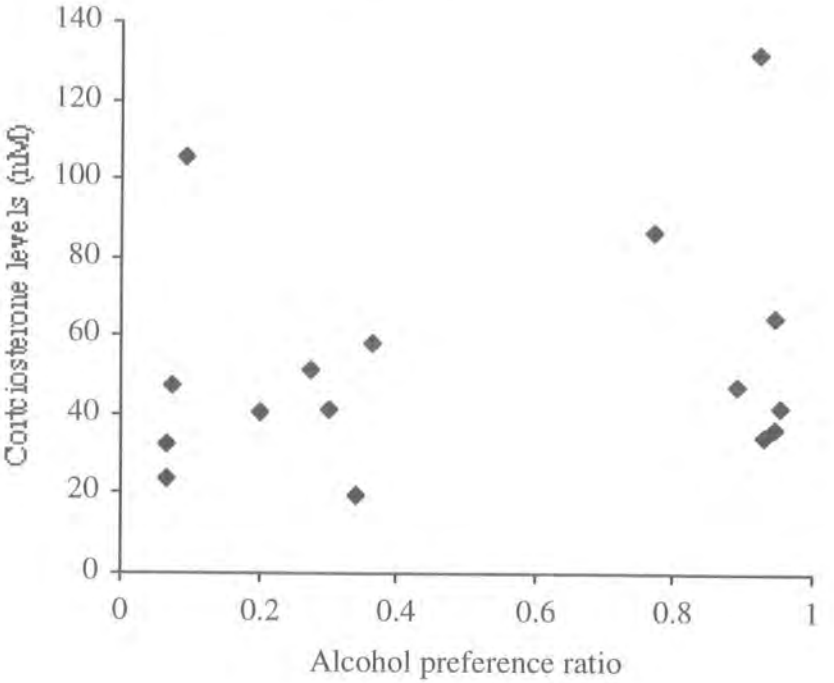


Figure 2.14 a. *Initial corticosterone levels and subsequent alcohol preference in C57 mice. The total plasma corticosterone levels were measured at lights on using the ICN supplied assay kit. There was no correlation between corticosterone levels and alcohol preference.*

Figure 2.14b

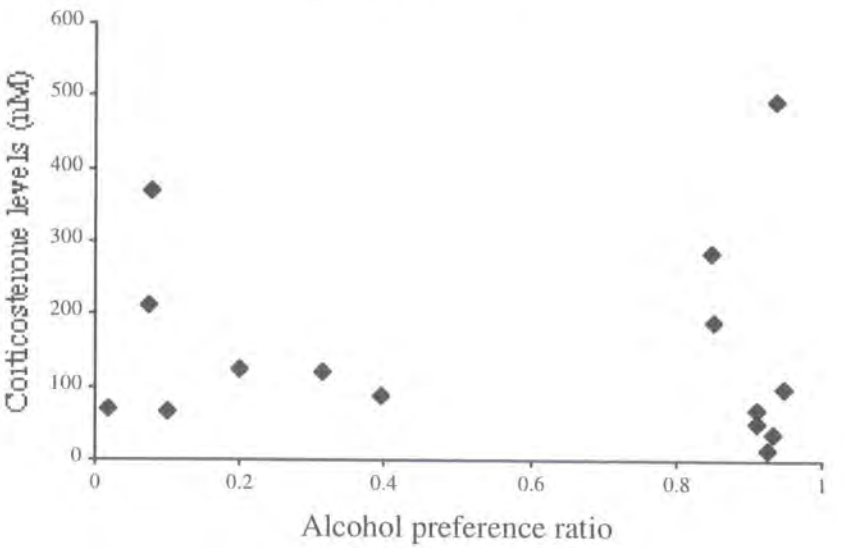


Figure 2.14 b. *Initial corticosterone levels and subsequent alcohol preference in C57 mice. The total plasma corticosterone levels were measured at lights off using the ICN supplied assay kit. There was no correlation between corticosterone levels and alcohol preference.*

Figure 2.15

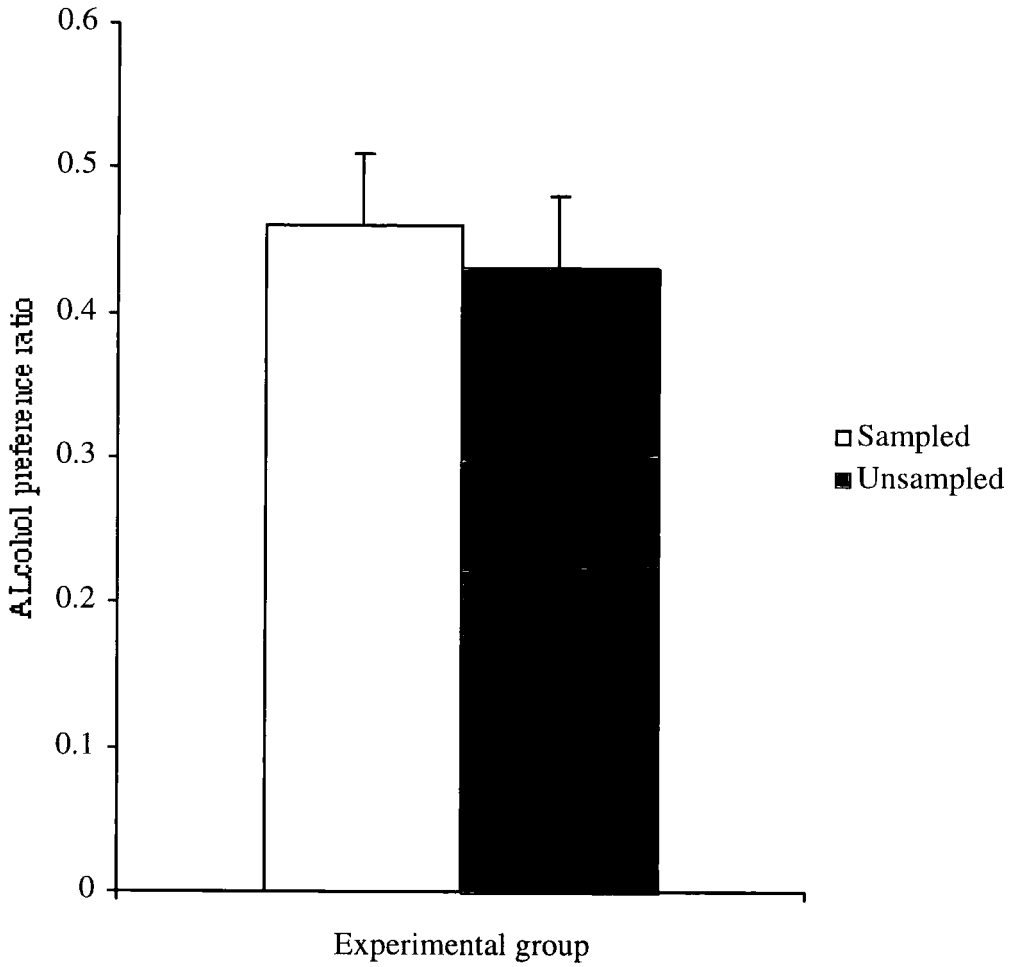


Figure 2.15. *The average alcohol preference of C57 that prior to screening had either blood samples taken (sampled) or were handled but no blood samples were taken unsampled (n=46 per group). The sampling procedure had no effect on subsequent alcohol preference (Mann-Whitney test P>0.5).*

Figure 2.16

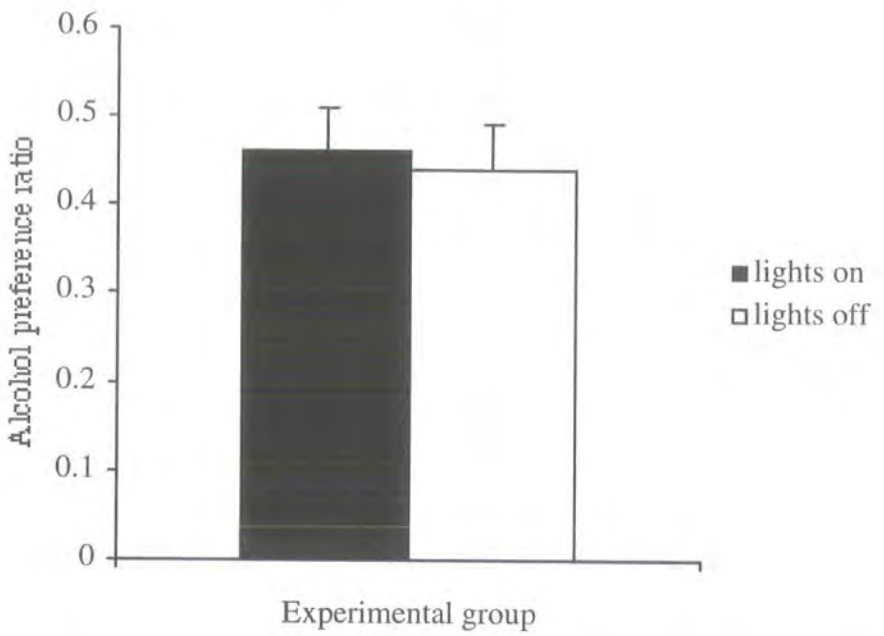


Figure 2.16 *Alcohol preference ratios of mice that were started on the alcohol screening procedure at either lights on, the start of the light phase, (n=46) or lights off, the start of the dark phase, (n=46).*

Corticosterone Assays

The discrepancy in absolute corticosterone levels recorded by the two methods has a number of possible explanations. A methodological explanation could explain part of the difference. The methods are broadly similar, i.e. RIA with a dextran/charcoal separation of antibody-bound and free tracer. The Sigma protocol, however, does not require a boiling step in sample preparation. Boiling the sample is used to destroy the plasma proteins aiding antibody/target binding. Although the Sigma antibody/corticosterone binding may be unaffected by the presence of plasma proteins, the binding of either endogenous corticosterone or added tracer to the plasma proteins which would be separated by the dextran charcoal in addition to the antibody tracer complex does not seem to be accounted for with this method, making a very real possibility of artificially high or low measures dependent on plasma protein content rather than corticosterone content. For this reason the ICN antibodies were used in all of the subsequent experiments in this thesis. The Sigma antibodies were also less stable than those from ICN.

Another possible reason for the difference in values may have been the treatment of the stock animals, although every care was taken to standardise the conditions in which the animals were housed both before and during any treatments. However, there is a possibility that the group that was assayed using the Sigma antibodies may have been fed a different laboratory chow. This is highly speculative, the only evidence for this is that the initial group contained animals that were over 35g, an excessive weight even for very mature C57 mice.

Since this experiment there have been no stock C57 mice that weighed over 35g. In the two experiments the experimental protocol varied slightly, this might also explain the difference in the measured values. The first experiment used blood samples that were taken from mice that had been moved from the holding rooms to another room to be sampled. This transfer may have been responsible for the higher measured corticosterone levels.

The two experiments did, however, produce comparable results even though the absolute levels that measured were different. Firm conclusions about the relationship between circulating levels of total corticosterone and alcohol preference could therefore be made on the basis of the results form both experiments.

C57 mice and alcohol preference

The C57 mice in these studies show the same bimodal distribution of alcohol preference that was originally uncovered in Bristol. This effect has been consistently shown over a number of years (Little et al., 1999), but the cause of this variation has not yet been identified. The high preferring mice can drink 15g/kg/day of alcohol. This is a large amount of alcohol even for high preferring strains of rodents; high preferring rat strains are normally reported to consume 6-9g/kg/day (Samson et al., 1998a, 1998b; Colombo, 1996). The low preferring mice can drink less than 1g/kg/day, an intake similar to other strains of low or alcohol avoiding rats (Samson et al., 1998a, 1998b). The relatively large amount of alcohol ingested by these mice compared with the high alcohol preferring rat strains may be explained by faster alcohol metabolism.

Locomotor activity and alcohol preference.

The ability of alcohol to increase locomotor activity is reported to be a measure of its rewarding efficacy (Pohorecky, 1981). The "psychomotor stimulant theory of drug addiction", proposed that the stimulant action of drugs of abuse is mediated by the same mechanisms that mediate reward and reinforcement. However, the relationship between alcohol and motor activation in various rat and mice strains is far from clear. Studies have found a relationship between alcohol administration and locomotor activity in some mice strains but not others (Cunningham et al., 1992). Rats of selected high preferring lines have been shown to exhibit a greater response to ethanol-induced locomotor activation than low preferring rats (Waller et al., 1990; Hilakivi et al., 1984).

There was no correlation between locomotor activity and alcohol preference for the whole population screened or for high or low subsections of the tested population. Testing of locomotor activity after alcohol preference screening also showed no relationship in these C57 mice (Little et al., 1999). Reasons as to why no correlation was found may be explained by the differences in responding measures rather than the rewarding aspects of alcohol compared to other drugs of abuse. This method measured alcohol preference, an established method of modelling some aspects of alcohol dependence, whereas the Piazza studies model rewarding behaviour through operant responding. These methods, although broadly answering some of the same questions, are very different. Preference to alcohol as measured by standard two bottle preference procedures does not correlate to operant responding for alcohol in all situations. A study by Samson and colleagues (1998a) have shown, using a variety of high and low alcohol preferring selectively bred rat strains, that preference does not correlate

to restricted access operant responding - where the animal is placed in the operant chamber for a limited amount of time. However, unlimited access operant responding does correlate to alcohol preference (Samson et al., 1998b).

The possibility that high responders and high locomotor activity. Reported in other cases, may in fact be measures of a similar underlying behaviour not directly related to high levels of drug self administration cannot be discounted. The enhanced locomotor activity is proposed to be a measure of an enhanced response to novel environments and therefore be linked to risk-seeking behaviour linked to drug abuse.

Another possible explanation is that some of the characteristics of the C57 mice may themselves be responsible for the lack of correlation. As stated in the introduction, many substrains of C57 mice in addition to the ethanol preference share other common patterns of behaviour including high levels of open field exploration, low levels of anxiety related behaviour in an open field, high learning capacity in many memory tasks (Crawley et al., 1997), and a high startle reactivity (Paylor and Crawley, 1997). It is possible that the inherent high locomotor activity amongst C57 mice prevented the observation of any potential relationship between activity and alcohol preference.

Corticosterone levels and preference

The lack of correlation between total corticosterone levels and alcohol preference was an unexpected finding given the reports of a relationship between the two (Prasad & Prasad 1995). The main difference between this study and the Prasad study was that they measured the excreted corticosterone levels and the present study measured the total blood corticosterone levels. In this set of experiments, which measured only the total corticosterone levels, it was not

possible to measure the free and bound corticosterone, as, at the time when the experiments were performed, the procedures were not available in our laboratory.

Corticosterone is excreted in urine and the levels found in urine are reported to be correlated to the levels of free corticosterone found in the circulation. Corticosterone is found in the circulation as either bound or free. Bound corticosterone describes corticosterone bound to a specific corticosterone binding globulin or other non-specific proteins in the blood. Normally the proportion of free corticosterone is about 10% of the total level. Under times of certain types of stress the proportion of free corticosterone rises dramatically (Savu et al., 1981). High levels of corticosterone in urine are linked to high circulating free corticosterone. The levels of total circulating corticosterone do not necessarily relate to the free levels. Free corticosterone is 'available' to its target sites either in the periphery or centrally. However, elevated total corticosterone levels have been demonstrated in response to stress and it is the total corticosterone levels that are generally discussed in the literature when examining a HPA response, although this might not be the most accurate measure of corticosterone response.

However, the link between free corticosterone levels and alcohol preference is not itself clear-cut. In their experiments, Prasad and Prasad investigated the basal and activated (levels after a stress) corticosterone levels and their link to alcohol preference in selected high preferring P rats and low preferring NP rats. They also examined the same relationship in unselected Sprague-Dawley rats. The comparison between the selected alcohol preferring strains and alcohol avoiding strains is limited because the strains are different. Multiple comparisons between many different strains are normally necessary to

gain meaningful data, hence a more complete investigation, would be necessary for a firmer conclusion. The suggested link between the alcohol preference of Sprague-Dawley rats and their corticosterone levels is valid because it is an intra-strain comparison. The significance of this correlation in the context of alcohol dependence or an individual susceptibility to alcohol dependence is less clear, as the alcohol preference of the rats was low (all the measured preference ratios being less than 0.4).

A specific relationship between circulating total corticosterone levels and alcohol consumption has not been established, but this does not exclude the relationship between the HPA axis and corticosterone. There are a multitude of possible sites and systems in the HPA that could modulate alcohol consumption. Some of these will be discussed and examined in later chapters.

There is more scope for investigation; the levels of free and bound corticosterone should be measured as total corticosterone levels present a limited picture of HPA axis activity. In addition these levels (free and bound corticosterone) could be measured in response to stress prior to preference screening rather than the baseline levels of total corticosterone could provide a more definite conclusion on the ability to predict alcohol preference by measuring HPA axis activity.

Chapter Three

Repeated stress and preference

Introduction

Studies have assessed the influence of both acute and chronic stress on drinking behaviour and the development of alcohol dependence. Alcohol drinking problems have been reported to be closely related to a variety of stressful experiences including illness, the death of somebody close, and from chronic occupational stressors, that were combined with a 'sense of powerlessness' (Seeman & Seeman, 1992). Crum et al., (1995) showed that men in high-strain jobs, those that had high demands and low control, had a higher risk of developing alcohol user disorders.

A relationship between stress and alcohol consumption in laboratory animals has become well established. Since the original observation by Masserman and Yum (1946), that 'neurotic' cats showed an increased preference for a mixture of milk and 5% alcohol to milk alone, numerous studies have been conducted examining the link between stress and alcohol consumption. The 'tension-reduction' hypothesis states that people drink alcohol in order to obtain the acute effect of relief of anxiety (Conger 1956, Williams 1966, Pohorecky 1981). However, Pohorecky (1981) has also argued that in humans alcohol can increase anxiety with time during a drinking binge. Alcohol consumption is reported to reduce the magnitude of an organism's response to stress (Levenson et al., 1980). Drinking to reduce stress, in individual cases may result in high consumption leading to, potentially, the development of dependence.

Increases in voluntary alcohol drinking after stress produced by immobilization has been reported (Rockman et al., 1987; Nash & Maikel, 1985), an effect that others have failed to demonstrate (Ng Cheong Ton et al. 1983; Fidler & Lolordo, 1996). Stress induced suppression of the maintenance, but not acquisition, of alcohol consumption in rats has been reported (Ng Cheong Ton et al. 1983). A consistent pattern has been the demonstration of increased alcohol consumption after these more severe stressors in low preference animals, with less, or no, effect on the preference of individuals with high preference prior to the stress. (Volpicelli et al., 1986; Rockman et al., 1987). The effect of stress is also seen in the self-administration of other drugs of abuse. Exposure to footshock stress potently reinstates heroin (Shaham & Stewart, 1995) and cocaine (Erb et al., 1996; Ahmend & Koob, 1997) self-administration. Acute stress facilitated self-administration only when drug availability was present a short time after the stress (Shaham, 1993) whereas after repeated stress animals showed enhanced self-administration of amphetamine even if the stress was absent for weeks (Shaham 1993, Pizza & Le Moal, 1998), indicating that persistent physiological changes occurred leaving the animal prone to enhanced self-administration.

Use of C57 mice

As previously stated alcohol is aversive to the majority of rodents. Although high preferring mouse and rat strains have been developed, their use in modelling alcohol consumption and dependence behaviour has some limitations, the most notable of which is, paradoxically, their consistent high voluntary alcohol consumption. When examining the factors that cause or are influenced by alcohol consumption, misleading weight may be given to discoveries that are

influenced unduly by a particular genotype. This can be illustrated by the Fawn-Hooded rat. The Fawn-Hooded rat will drink large quantities of alcohol and is described as a high alcohol preferring strain of rat. The functioning of the CNS serotonergic system in this type of rat is abnormal compared to outbred strains. The influence of 5HT on alcohol consumption is well established, however using such a strain will provide only (more) evidence on the links between 5HT and alcohol consumption. In the 'high preferring' C57 mice, however, the existence of low preferring mice allows a greater scope for investigation (see chapter two). This enables the investigation of environmental or pharmacological factors that alter alcohol preference that are independent of, and not constrained by the influence of a particular genotype that already defines the strain. Previous work with these mice has shown that mild environmental disturbances such as moving the mice down several floors in a lift (unpublished data) increase alcohol preference.

Repeated saline injections increased the alcohol preference of low preferring C57 mice (Little et al., 1999). Low and high preferring mice were injected with the CCK_B receptor antagonist CAM 1028, to investigate potential actions of this novel anxiolytic on alcohol consumption. This was shown to have no effect on alcohol preference when administered to high alcohol preferring, and no 'apparent' effect on low alcohol preferring, mice. However, the repeated saline vehicle injections in the low preferring mice caused an increase in preference Antagonists at the CCK_B subtype of receptor have been reported to decrease anxiety-related behaviour in animal tests, although differences in the results of studies on this have been reported (Hughes et al., 1990; Singh et al., 1991a; Dawson et al., 1995). These drugs are effective in decreasing anxiety-

related behaviour in mice when such behaviour is increased during the acute phase of alcohol withdrawal (Wilson et al., 1998), but had only small protective action against the convulsive aspects of the alcohol withdrawal syndrome (Wilson & Little, 1998).

Those results suggested that the actions of the CCK_B antagonist in preventing the increase in alcohol consumption caused by minor stress in low preferring C57 mice was due to an interaction with the neuronal changes involved in the response to the minor stress, rather than a direct effect on the mechanisms that control alcohol drinking in the absence of stress.

Aims

The general aim of this section of experiments was to investigate the effects of repeated mild stressful procedures on alcohol preference in low alcohol preferring C57 mice. Initially the effects of diazepam on alcohol preference in low preferring mice were examined. This was performed in order to discover whether the effect of injection induced increases in alcohol preference, that had been shown to be inhibited by CAM1028, could be inhibited by the anxiolytic drug diazepam. Also an examination of the effects of repeated dilute tween vehicle injections, saline injections, or needle insertion (but no injection) compared to handled (but not injected mice) was performed to uncover whether the injection procedure itself or the disturbance of handling was responsible for the increase in alcohol preference. Single saline injections were given to mice to see if the increase in preference was a function of time or if the repeated stress was necessary for the increase. Repeated saline injections in the absence of alcohol were performed to discover whether the increase in preference was dependent on the availability of alcohol.

Methods

Male C57 mice were used for all the following experiments. The conditions, housing, and alcohol screening procedure have been described in Chapter Two. The mean alcohol preference ratios for the last week of measurements (the third week, when the measured ratios were stable) were used to allocate mice to the preference categories. Mice with a ratio of 0.7 and above for the consumption of 8% alcohol over water were classed as "high" preference, and those showing a ratio of below 0.3 were classed as "low" preference. The following experiments were performed on male C57 mice that had undergone the screening procedure and were designated low preferring mice i.e. their alcohol preference was below 0.3 during the last week.

Effects of vehicle and diazepam injections

A study was carried out to examine whether the handling of the mice or the act of injection plus the handling was the crucial factor in the effects of the saline injections. Parallel groups of male mice (25-30g, n=9), were either picked up, positioned ready for injection then returned to the home cage or picked up, positioned and injected with (1µl/kg) 0.9% saline. These treatments were continued for 3 weeks daily and measurements of alcohol preference were made three times per week (1p.m - 2p.m. Monday, Wednesday and Friday) throughout the experiment. The treatment schedule of three weeks was chosen because the saline induced increase in alcohol preference was first observed (Little et al., 1999) after 10-15 days. A three week treatment schedule should, therefore provide sufficient time for any alterations in preference to develop.

The effects of diazepam (1 mg/kg, i.p.) were examined in a further study on mice previously screened and classed as low alcohol preferring animals. This dose of diazepam was chosen as it had previously been shown to reduce anxiety related behaviour on the elevated plus-maze (Cole & Rodgers, 1995). Three parallel groups of mice were used: one was injected with diazepam, 1 mg/kg (male mice 25-30g, n = 7), the second with the tween vehicle (male mice 25-30g, n = 8), and in the third (male mice 25-30g, n = 9) the animals were picked up, positioned ready for injection then returned to the home cage. The tween vehicle was a suspension of 0.05% tween in saline. Tween was used as the vehicle in these injections as diazepam is insoluble in water or other polar solvents, so it was necessary to administer the diazepam in a detergent suspension. Measurements of alcohol preference were made once daily for three weeks at 2.p.m. All the mice were weighed once a week (Wednesday).

Repeated /single injection

This experiment further examined the previously demonstrated phenomenon that daily intraperitoneal injections of isotonic saline increased the alcohol preference of low preference mice when given over a period of three weeks. Also examined was the effect of a single saline injection on alcohol preference when monitored for three weeks after the injection. The mice (male mice 25-30g, n=8) were injected once daily with isotonic (0.9%) saline (0.001ml/kg) every day for three weeks or were handled as if to be injected but placed back into their cage without injection (male mice 25-30g,n=8) or were injected on the first day only (this group was left undisturbed except for bottle weighing). These treatments were continued for 3 weeks and measurements of alcohol preference were made three times per week (1p.m - 2p.m. Monday,

Wednesday and Friday) throughout the experiment. All the mice were weighed once a week (Wednesday).

Injections without alcohol available

Two groups (25-30g, n = 8 per treatment group) of low preference male mice were continued on single housing and given tap water as the sole available fluid and either (i) handled daily as if an injection was going to be made, but not injected or (ii) injected once daily with (10ml/kg) isotonic saline, by the intraperitoneal route. These procedures were carried out at 2 p.m. each day. At the end of the three week period, the animals were provided with a free choice between 8% v/v alcohol and tap water and fluid consumption measured, three times per week (Monday, Wednesday and Friday) for the week immediately following the procedure. All the mice were weighed once a week (Wednesday).

Sham injection and alcohol preference

Two groups (male mice 25-30g, n = 8 per treatment group) of low preference male C57 mice were continued on single housing and provided with a free choice between 8% v/v alcohol and tap water for the duration of the experiment. For three weeks, they were either (i) handled daily as if an injection was going to be made, but not injected or (ii) received an intraperitoneal insertion of a syringe needle but no injection of fluid ('sham injection'). These procedures were carried out at 2pm each day. Fluid consumption was measured three times per week (Monday, Wednesday and Friday) for the three weeks of the treatment. All the mice were weighed once a week (Wednesday).

Statistical analysis

All data is presented as mean ±SEM. *A priori* assumptions enabled the comparison between treatments (at three weeks) and baseline values to be performed using t-tests in all the experiments except the 'sham' injection experiments where analysis of variance and when appropriate Fisher's post-hoc test was used. Significance was set at $P<0.05$.

Results

Injections and preference

The results from the study comparing the alcohol preference of mice, previously classed as "low preference", after either handling or saline injections, are illustrated in Figure 3.1a. During the third week of treatment, the group, which received the injections, showed significantly higher preference than those that were just handled ($P < 0.02$). No changes were seen in the total fluid consumption of either group during the saline administration. Figure 3.1b illustrates the alcohol preference of mice, previously classed as "low preference", after diazepam (1 mg/kg), Tween vehicle, or handling alone. By the third week, the alcohol preference after Tween injections or diazepam showed significant increases from baseline (both $P < 0.01$) but there was no effect of handling compared with the corresponding baseline ($P > 0.1$). Within the third week, the mean preference after Tween injections was higher than that after handling, but the difference did not reach significance. The difference between diazepam and handling was significant, but there was no significant effect of diazepam compared with Tween vehicle. The administration of diazepam had no significant effects on the alcohol preference, compared with injection of its vehicle ($P > 0.1$). No changes were seen in total fluid consumption of any group during the drug or vehicle administration.

Figure 3.1a

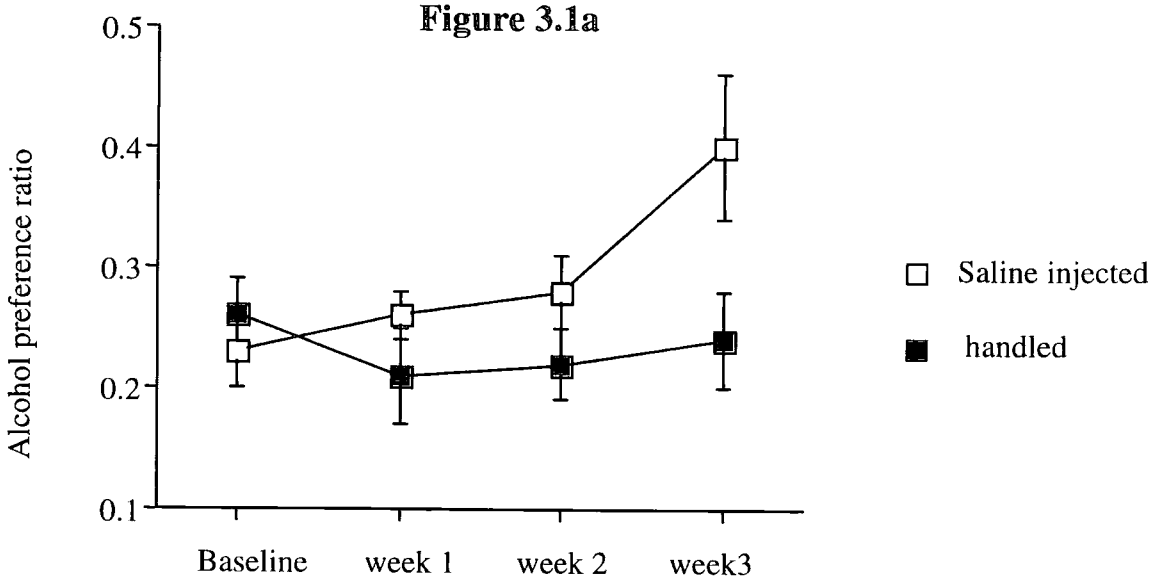


Figure 3.1b

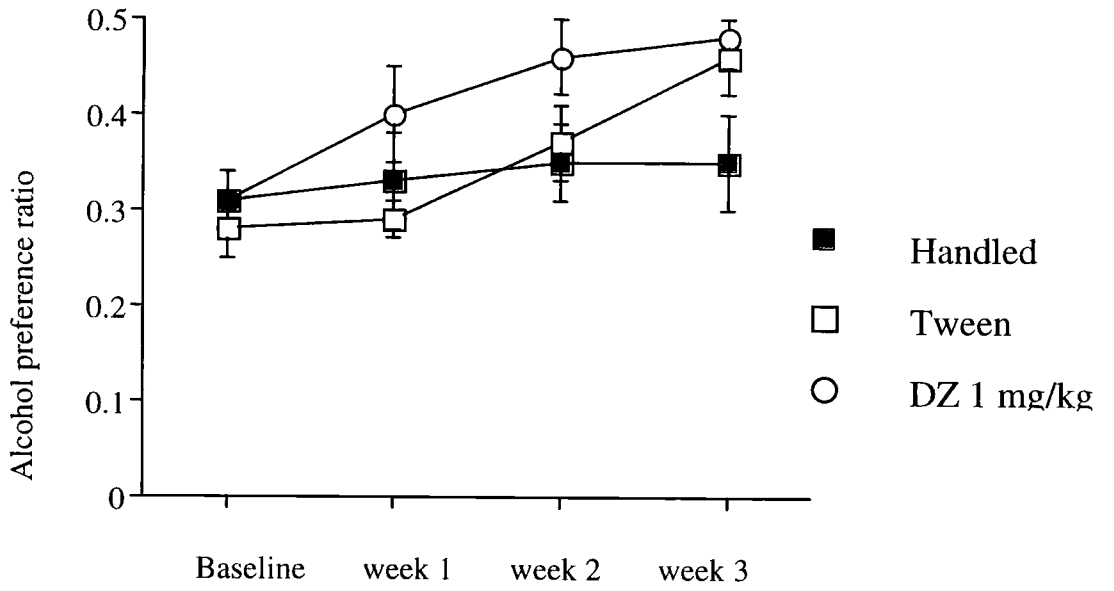


Figure 3.1a *The change in alcohol preference, over three weeks in saline injected C57 mice (filled boxes) compared with handled only mice (open boxes) (* P<0.02).* **Figure 3.1b** *Increased alcohol preference in tween and diazepam (DZ) injected mice compared to baseline (** P<0.01).*

Repeated or single daily injections with access to alcohol.

A single injection of saline did not alter alcohol preference in low preferring mice, compared either with pre-treatment baseline preference values or the handled only controls, when measured three weeks after administration of the injection, the time point at which multiple saline injections were shown to increase alcohol preference. Repeated saline injections, as in the previous study increased alcohol preference when compared both to the handled only mice, $P<0.05$ and the pre-treatment baseline $p<0.05$. The baseline being the mean of the final three measures in week three of the screening. Repeated saline injections also increased alcohol preference in mice compared to those that received a single saline injection $P< 0.05$(Figure 3.2).

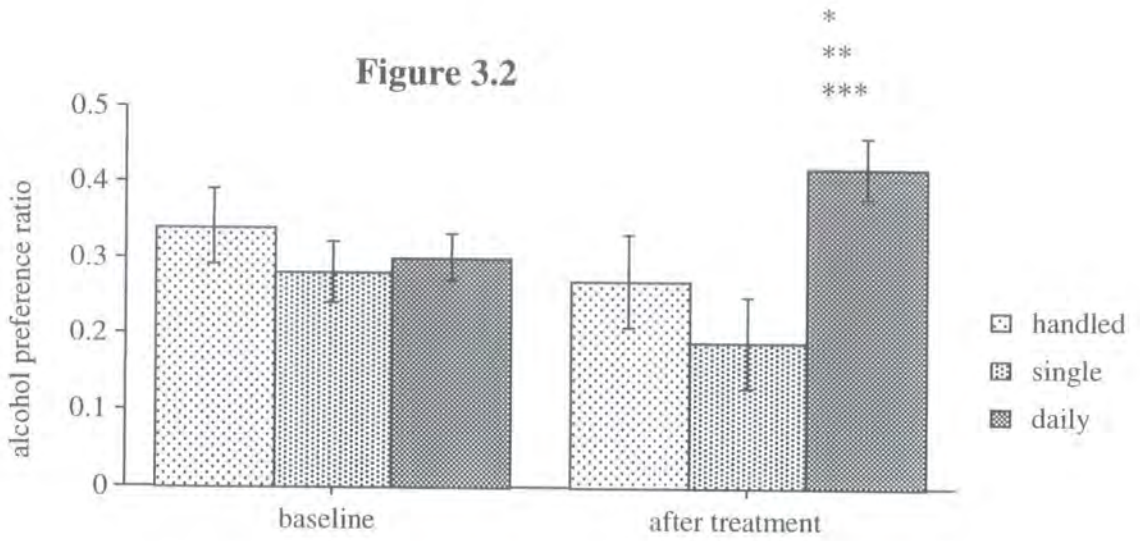


Figure 3.2 *The mean alcohol preference in mice before (baseline) and after receiving a single saline injection, daily saline injections or handling alone. Results are the mean preference ±SEM of the third week of treatment (n=8 per group). *P<0.05 daily injections vs. baseline; **P<0.05 daily injections vs. handled; ***P<0.05 daily injections vs. single.*

Repeated saline injections without access to alcohol

The administration of daily i.p. injections of saline increased the alcohol preference of low preference mice, when access to alcohol was available only at the end of the injection schedule (Figure 3.3). The alcohol preference ratio was significantly higher in those mice that received the saline injections, both compared with the baseline measured prior to the injections ($P < 0.0001$) and compared with the alcohol preference of mice that received only handling during the three weeks ($P < 0.001$). Total fluid consumption was unchanged by either treatment.

Figure 3.3

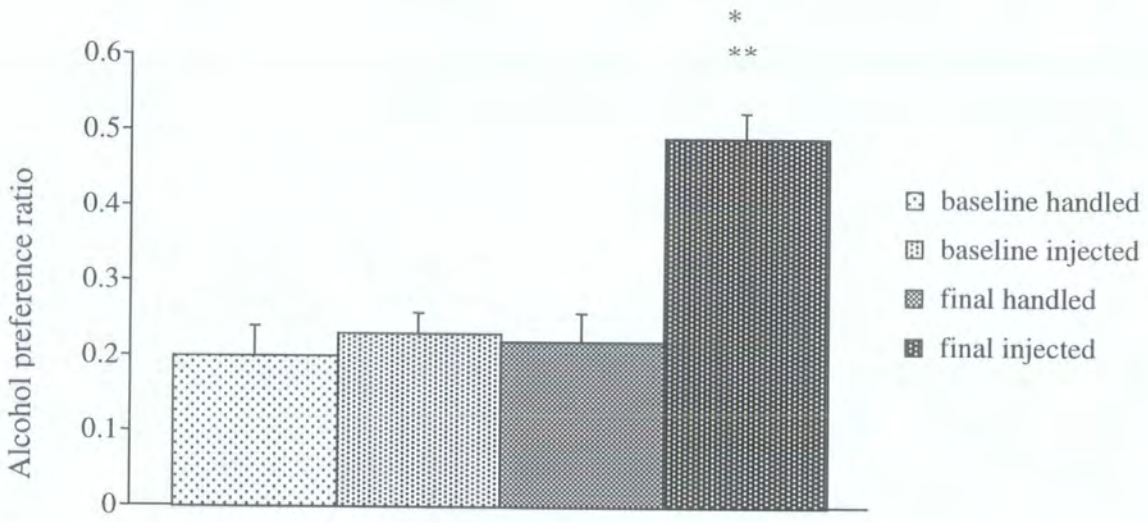


Figure 3.3 *Subsequent alcohol preference in previously identified low preferring mice that received, daily saline injections or were only handled without access to alcohol. Results are the mean preference ± (n=8 per group). *P<0.001 saline injections vs. baseline; **P<0.01 saline injections vs. handled final.*

Sham injected and alcohol preference

The sham injections significantly increased the alcohol preference of low preference mice ($F_{5,30} = 5.42$, $P = 0.0089$) compared with the preference of the parallel group of mice that were only handled ($P < 0.001$ for week 2 and $P<0.01$for week 3) and compared with the baseline preference ($P<0.01$ for two weeks and $P<0.05$ for the third week) of the mice (Figure 3.4). Total fluid consumption was unchanged by either treatment. In contrast to previous experiments the sham-injected mice showed an increase in alcohol preference in the second week of treatment, rather than the third week seen in the saline injected. For this reason the measurements form the second week of treatment are included in the figure below.

Figure 3.4

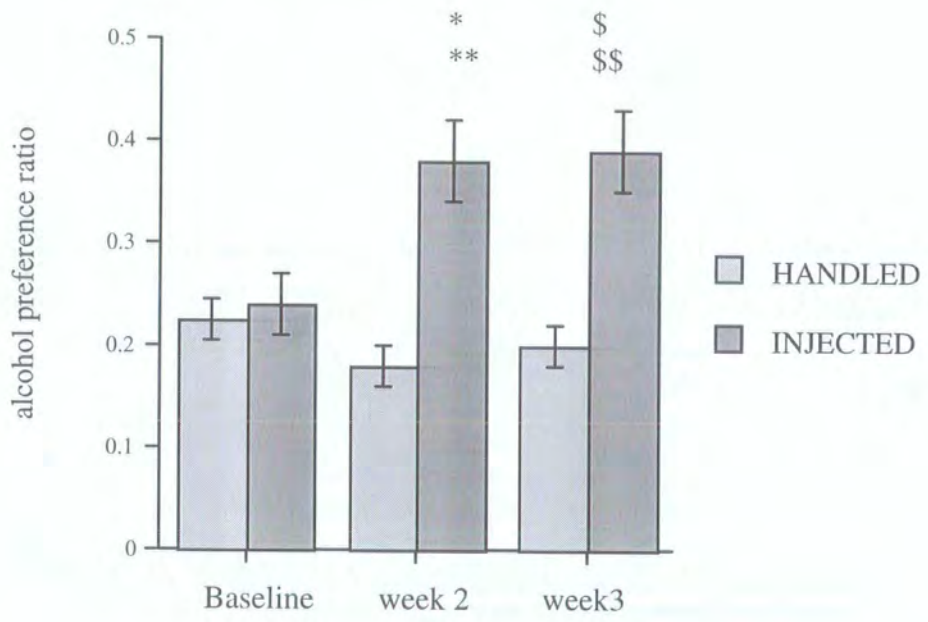


Figure 3.4 *Weekly alcohol preference ratios in mice that were handled or received a daily sham injection. Daily sham injections increased alcohol preference, at week two compared to the baseline measures (*P<0.01) and handled mice (**P<0.001), this increase was maintained at week three compared to baseline ($ P<0.05) and handled only mice ($$ P< 0.01).*

Discussion

The results have demonstrated that daily injections of saline significantly increased the alcohol preference ratio of low preference mice. Handling the mice without injecting them did not increase alcohol preference. It was not necessary for alcohol to be available during the screening procedure for an increase in preference to develop nor was it necessary for any fluid to be injected to increase preference. Saline injection induced increases in alcohol preference are not confined to Durham C57 mice; increases in alcohol preference have been reported in rats following six days of injection (Stromberg et al., 1997). The tween vehicle injections caused an increase in alcohol preference. An increase in alcohol preference was also seen after repeated injections of diazepam at a dose previously shown to be anxiolytic (Cole & Rodgers, 1995).

Other mild stimuli (O'Callaghan et al., 2001) have been shown to increase the alcohol preference of low preferring C57s. The movement of these mice, by lift, down seven floors (between laboratories at Bristol Medical School) increased the alcohol preference of low alcohol preferring mice. This increase in alcohol preference was only seen two months after the move. However, not all disturbances appear to increase preference. Moving cages of animals between rooms did not alter preference even though this procedure may increase corticosterone levels (see Chapter Two). Neither exposure to ultrasound nor repeated cage cleaning altered the preference distribution. In those cases alcohol preference ratios were measured over three weeks after the disturbance (Watson, Cole & Holt unpublished data). Alcohol preference in defeated low alcohol preferring C57 mice (from a resident intruder situation) was increased, but only after repeated defeats (five days). Single defeats or repeated exposure to a novel

cage did not alter alcohol preference (Holt, 2001). All these manipulations can be described, as 'stressful' whether they can be ranked according to the severity of stress is debatable. The common thread when a stressor alters preference appears to be the repetitive nature of the stress (except in the case of the movement of the animals via a lift). The type of stress also appears important. Stressors that comprise a physical component such as electric footshock and tail-pinch induce an increase in self-administration of drugs of abuse. Other stressors lacking a physical component, e.g. decapitation of conspecifics, can also enhance self-administration. Decapitation of conspecifics also results in increased in CCK_B receptor binding that is also seen after repeated saline injections (Harro et al., 1996).

Penetrating the mice with a needle but without injecting any fluid was performed to ensure that the increase in preference was not caused by any physiological change in fluid balance. An injection of 10ml/Kg is a substantial amount of fluid to give to a mouse i.p and may have influenced the fluid balance of the animal, although this is the normally used (in our laboratory) volume for administering drugs. Prior to the sham injections it was postulated that perhaps the stressor might not have been the injection but the temperature of the saline or tween. Normally injections are performed with the temperate of the solutions at approximately room temperature. Given that a mouse's core temperature is 37-40°C a volume of fluid at 21^0C injected into the body cavity could activate a number of vigorous homeostatic processes to maintain the normal core temperature. These homeostatic processes could have been responsible for the increase in alcohol preference.

The studies on the saline injection procedure demonstrated that it was not necessary for alcohol to be available during the experience for the increase in alcohol preference to be seen. It was also unnecessary for saline to be injected, since the sham injection procedure also had the effect of increasing the alcohol preference, with a similar time course to that seen in the original demonstration of the effect of the saline injections (Little et al., 1999). This suggests that the effect is due to the stress of the procedure.

Perhaps the two most important aspects of these results were firstly that the effects of disturbance in increasing alcohol preference were slow in onset, and, secondly, that the disturbance that increased the preference was relatively mild in comparison with some of the stressors used in other laboratories that alter alcohol consumption. These results have important implications for studies on this strain. Firstly, injections of saline or drug vehicles are normally considered routine procedures without behavioural consequences, so the effects demonstrated in the present studies may be an important, previously unrecognised, factor. In addition, animals bought from outside sources undergo, for example, the stresses of transport before they reach laboratories. It is possible that while the animals used in many laboratories show high alcohol preference, they may not have begun life with such preference.

The effects of the minor stress in increasing the alcohol preference of low preference C57 mice provides a valuable model for the study of the mechanisms involved in increases in alcohol consumption. An important aspect is the nature of the change in the alcohol consumption, whether, for example, involves alterations in the rewarding, or aversive, effects of alcohol. Further studies on this model are in progress to investigate these aspects.

An advantage of this model is that the stress applied is not excessive. Major stressful experiences, such as restraint or footshock, have a profound effect on the metabolism and brain neurochemistry and are therefore likely to cause many changes some of which may not be related to alterations involved in alcohol consumption. Stress such as restraint can inhibit a large proportion of the normal behavioural repertoire, but the present model does not affect the normal behaviour, such as eating or drinking of water, and alters only the alcohol consumption.

The saline injections were described as stressful and measurement of corticosterone levels, both circulating and brain, were elevated in injected compared with handled mice (see Chapter Five). Although the increased alcohol preference by vehicle injections was consistently demonstrated, and an increase in corticosterone levels was shown after single saline injections there is no evidence that the two factors are linked.

Chapter Four

The action of steroid antagonists, ACTH, CRF and metyrapone on alcohol preference in C57 mice.

Introduction

The experiments in previous chapter show a link between stress between and alcohol consumption, however, in Chapter Two no relationship between total corticosterone levels and alcohol preference was found. This finding does not necessarily refute the wealth of experimental evidence that reports a link between corticosterone and alcohol consumption (e.g. Fahkle & Eriksson, 2000). Activation of the HPA axis (the stress response) is described in detail in Chapter One. Briefly, following stress, CRF is released; this stimulates the release of ACTH that in turn stimulates the synthesis and release of corticosterone. Release of corticosterone, CRF and ACTH is controlled by negative feedback (see Figure 1.2). Following the results from chapter one it is proposed that the control of alcohol consumption by stress may be a result of the negative feedback control on levels of CRF and ACTH rather than a direct effect of corticosterone itself. In this chapter the influence of the various major components of the stress response (ACTH, CRF and corticosterone) on alcohol consumption were examined.

It has previously been shown that both surgical adrenalectomy and inhibition of corticosterone synthesis by administration of the corticosterone synthesis inhibitor metyrapone reduces alcohol consumption in rats (Fahkle et al., 1995; 1994b). The levels of total circulating corticosterone were not

correlated to alcohol preference in C57 mice (Chapter Two). Metyrapone was administered to high alcohol preferring C57 mice to examine whether a reduction in circulating levels of corticosterone could reduce alcohol consumption.

Corticosterone and corticosterone receptors.

Most of the effects of corticosterone are believed to be mediated via its intracellular receptors. These receptors are typical steroid hormone receptors. This family includes the receptors for all the steroid hormones (other examples include oestrogen, androgen and progesterone receptors). Corticosterone binding to the receptors produces translocation of the activated hormone–receptor complex to the nucleus where it interacts with specific DNA sequences to produce its effects on metabolism and homeostasis.

There are two types of corticosteroid receptor (Reul & de Kloet, 1985): the mineralocorticoid (MR, type I corticosteroid) receptor and the glucocorticoid receptor. The glucocorticoid receptor (GR, type II corticosteroid receptor) is a polypeptide chain of about 780 amino acids (Hollenberg et al., 1985). Unoccupied GR is present in the cell cytoplasm as part of a heterocomplex comprised of three different heat shock proteins. These proteins play a key role in the receptors' ability to be activated by the ligand. The GR has a lower affinity for corticosterone than MR, with a Kd of 5-10nM, but it shows a high affinity for the synthetic steroid dexamethasone (Kd ~ 1nM) (Reul and de Kloet, 1985). The MR, structurally similar to the renal mineralocorticoid receptor, has a tenfold higher affinity with Kd's for corticosterone and aldosterone of between 0.5 and 1 nM (Reul and de Kloet, 1985).

Ligand-activated corticoid receptors attach to specific DNA sequences, classically as homodimers, however GR and MR can form

heterodimers (Rupprecht et al., 1993). These may be more potent than homodimers of MR or GR for DNA binding and gene transcription. The proportion of homo to heterodimers produced depends on tissue region and the availability of ligand. Some researchers have proposed that there is an additional membrane-bound low affinity corticosterone receptor although most of the evidence has been produced by work on newts (Orchinik et al., 1988; Orchinik & Murray, 1994; Orchinik & McEwan, 1994; Orchinik, 1999). Sze (1996) has described a low affinity (Kd 100nM) binding site in rat brain synaptic membranes.

ACTH and pro-opiomelanocortin related peptides

ACTH is a 39-aminoacid straight chain peptide derived from pro-opiomelanocortin (POMC). This is the precursor for ACTH, and α,β and γ melacortin secreting hormone (MSH), as well as β-endorphin. At times of stress, CRF stimulates the release of ACTH from the hypothalamus, POMC is cut by a series of post-translational proteolytic cleavages and ACTH (as well as β-endorphin) is released. ACTH stimulates corticoid production and secretion in the adrenal glands and lipolysis in adipocytes.

Hypothalamic neurons contain most of the brain POMC related peptides. However, ACTH is also found in the amygdala, cerebral cortex, brain stem, and cerebellum - even after ablation of the hypothalamus. This suggests that ACTH is synthesised in other brain regions in addition to the hypothalamus (Civelli et al., 1982; Romangnano & Joseph, 1983; Schwartzberg & Nakane, 1983; Pilcher and Joseph, 1984; de Souza et al., 1985). A number of functionally active ACTH fragments have been identified e.g. $ACTH_{4\text{-}10}$, $ACTH_{1\text{-}13}$, and $ACTH_{18\text{-}39}$, also

known as CLIP (or corticotrophin-like intermediate lobe peptide) and $ACTH_{1-13}$. Fragments of ACTH have been shown to have differing activities for steroid biogenesis; $ACTH_{1-24}$ and $ACTH_{5-24}$ are full agonists, whereas ($ACTH_{7-23}$) is a partial agonist and $ACTH_{7-25}$ is an antagonist (Lin et al., 1991). $ACTH_{5-9}$ is required for full receptor activation; $ACTH_{1-4}$ and $ACTH_{10-20}$ have greater peptide receptor affinity (Lin et al., 1991).

Only a small proportion of systemically administered ACTH is reported to enter the brain, about 1-2%. Estimates of systemically injected $ACTH_{4-10}$ report about 0.01% of the amount injected penetrates the brain (Potaman et al., 1991). However, 0.04mg/kg $ACTH_{4-10}$ has demonstrated anticonvulsant activity in pilocarpine induced seizures when injected i.p. (DeWied & Wolterink, 1988). $ACTH_{4-10}$ is also devoid of any adrenal activating properties (DeWied & Wolterink, 1988) i.e. this fragment does not stimulate either the synthesis of, or the release of corticosterone.

ACTH and ACTH fragments interact with multiple receptor subtypes. ACTH shows activity at the melanocortin receptors (MC-R) MC1-R (involved in pigmentation), MC2-R (adrenal function), MC3-R (cardiovascular regulation), MC4-R (energy homeostasis) and MC5-R (exocrine secretion). Overlapping sequences in the POMC molecule for ACTH and the various types of MSH as well as the activity of other ACTH fragments produce an array of actions that make a definitive functional assessment difficult (Pranzatelli, 1994).

ACTH and alcohol consumption

Alterations, either endogenous or pharmacologically prompted, in corticosterone levels will result in reactive alterations in other areas of the HPA.

The relationship between alcohol consumption (and drug self-administration) and ACTH has not been investigated fully. Rats will self-administer ACTH intravenously (Johaneau-Bowers & Le Magen, 1979), and it has also been shown that acute injections of ACTH (10mg/kg) increase the rates of lever pressing during extinction of food-reinforced behaviour (De Weid & Jolles, 1982). Administration of $ACTH_{4-10}$ in high consuming rats has been reported to reduce alcohol consumption (Krishnan et al., 1991).

Corticotrophin releasing factor (CRF)

CRF, a neuropeptide secreted by hypothalamic and extra-hypothalamic neurons, is thought to mediate stress-related behaviours. Hypothalamic CRF facilitates the release of ACTH from the anterior pituitary, and ACTH in turn elicits the release of glucocorticoids from the adrenal cortex. CRF binding sites are extensively distributed throughout the brain (De Souza, 1995). CRF binds two subtypes of CRF receptor (CRFR), CRFR1 and CRFR2. The latter exists as two splice variants, the neuronal CRFR2 alpha and the peripheral CRFR2 beta. CRFR is a G protein-dependent receptor which acts mainly through G(s) enhancing cAMP production (Eckart et al., 1999). Besides the two receptors, a 37 kD CRF binding protein (CRF-BP) binds several CRF peptides with high affinity (Eckart et al., 1999). CRFR and CRF-BP do not share a common amino acid sequence representing the ligand binding site CRFR1 is not only involved in the hypophyseal stimulation of ACTH release, but hippocampal CRFR1 mediates enhancement of stress-induced learning. CRFR1 may also be involved in basic anxiety. In contrast, at least in the mouse, CRFR2 of the lateral intermediate septum mediates tonic impairment of learning (De Souza, 1995). In response to stressful stimuli or after local injection of high CRF doses, CRFR2 mediates anxiety (De Souza, 1995). Several studies have identified a relationship between

CRF and factors associated with alcohol intake. One in vitro study showed that alcohol administration had a direct stimulatory effect on CRF release from rat hypothalamic organ culture system (Redei et al., 1988). In rats increased levels of CRF mRNA have been shown using in situ hybridisation following intravenous or i.p. injections of alcohol (Rivier et al., 1990; Rivier & Lee, 1996). It has been reported by Bell and co-workers (1988) that intra cerebroventricular (i.c.v.) injections of CRF reduced alcohol intake in a limited-access (1h/day) model of voluntary alcohol drinking.

CRF has anxiogenic actions and CRF antagonists have been reported to show anxiolytic properties. Anxiogenic-like responses on the elevated plus maze (reductions in % time spent on the open arms of the maze and reductions in the % open arm entries) are seen in rats during alcohol withdrawal. The CRF antagonist α-helical CRF $_{9\text{-}41}$ when given i.c.v. has been shown to reverse this anxiogenic-like response (Baldwin et al., 1991)

Aims

This study examined the effects of inhibition of corticosterone synthesis in high preferring C57 strain mice. The work aimed to examine the effects of selective corticosterone type I and II receptor antagonists in order to classify the interaction between corticosterone and alcohol consumption with respect to a particular receptor subtype. Administration of a corticosteroid receptor antagonist would be expected to reduce alcohol preference if corticosterone receptors were involved in the mechanism governing stress induced alcohol consumption. Reducing the levels of corticosterone will reduce the negative feedback on both CRF and ACTH secretion, resulting in increasing levels of both these peptides. These increased levels of peptides may be responsible for alterations in alcohol consumption induced by stress and so the effects of the CRF antagonist α-helical CRF $_{9\text{-}41}$ and the ACTH fragment, ACTH $_{4\text{-}10}$, on alcohol intake in low and high preferring mice were investigated.

Methods

Drugs used

Metyrapone reduces cortisol and corticosterone production by inhibiting the 11-β-hydroxylation reaction in the adrenal cortex. The apparent half-life of elimination averages 1 to 2 1/2 hours. Within 2 days after initiation of treatment, about 40% of the administered dose is excreted in the urine, mostly in the form of glucuronides (IUPharm, 2001)A 50mg/kg dose of metyrapone was chosen for this study as this dose had previously been reported to reduce alcohol consumption in high preferring rats.

Spironolactone and **RU38486.** A number of specific antagonists exist for both mineralocorticoid receptors (e.g. spironolactone) and for glucocorticoid receptors (e.g. RU38486). The i.c.v. infusion of selective antagonists allows definition of receptor specific corticosterone receptor mediated events in intact rats (De Kloet, 1991). The central administration of these drugs was chosen in preference to systemic administration so that any actions that might have been recorded could be attributed to direct central effects without the complications of any peripheral actions.

α-Helical CRF is CRF receptor antagonist that binds both receptor CRF subtypes (Eckart et al., 1999). This drug has previously been shown to reduce alcohol preference and fluid consumption in rats when administered (i.c.v. 5μg) to rats (Bell et al., 1998).

ACTH $_{4\text{-}10}$. ACTH $_{4\text{-}10}$ is a fragment of ACTH that has been shown to reduce alcohol consumption in rats at a dose of 10mg/kg (Krishnan et al., 1991).

This study used the same dose for this reason. The fragment particular fragment was also chosen as it lacks steroid synthesising and releasing effects (DeWied & Wolterink, 1988) and so interpretation of any results would not be complicated by ACTH stimulated corticosterone release.

Alcohol preference measurements

All tests of preference were made on male C57 (25-30g) mice. The mice were screened for alcohol preference (as previously described). High preferring mice (those with an 8% alcohol to total fluid ratio of greater than 0.7) and low preferring mice (those with an 8% alcohol to total fluid ratio of less than 0.3) were selected for subsequent experiments.

Metyrapone injections

Metyrapone was given, by the intraperitoneal (i.p.) route (50mg/kg in the first instance) to male high preferring C57 mice (25-30g n=6 per group), each evening at 5 p.m., with controls receiving vehicle 0.05% tween (see Chapter Three). The amounts of 8% alcohol and of water consumed were measured at 10 p.m. the next day. This experiment was then repeated, with the drug dose increased to twice daily injections (9 a.m. and 5p.m) of metyrapone (100mg/kg) (n=6 per group). Alcohol preference was measured at 4 p.m. for seven days. The increased dose (100mg/kg) was used to reduce the possibility that the lack of effect of the lower dose was due to faster metabolism in mice.

I.C.V. injection procedure

Drugs were administered to the unanaesthetised mice by the i.c.v. route using a custom made holder for the head through which a needle could be guided to pierce the skull. The mouse's snout was fitted into a rectangular holder with adjustable flanges and a collar that fitted round the neck, thus holding the neck

secure. A block was mounted on the holder, with a guide hole, to position the injection needle into the third ventricle; the distance from the collar to the guide hole was 6 mm. The needle was a steel cannula (27 gauge) through which microlitre volumes of drug could be injected. The mouse was held firmly by the neck collar while the drug was injected (2µl volume) into the third ventricle. The process took no more than thirty seconds. Immediately after release most of the mice appeared dazed for a short time (5-20 seconds). If any mice did not return to apparently normal behaviour after more than thirty seconds they were excluded from the experiment (n=1).

Corticosterone antagonists

To male C57 mice (25-30g) either 0.05% tween vehicle, 150ng of RU38486 or 150 ng spironolactone was given by the intracerebroventricular (i.c.v.) route (2µl volume), using a stereotaxic apparatus that enables injection in conscious mice (n=8 per group). The injection was given at the start of the dark phase (8 p.m.). The doses of antagonists were chosen because they have both been shown to be effective doses in reducing anxiety-like behaviours on the elevated plus maze (RU 38486) and in the black box / white box test (spironolactone) (De Kloet, 1991). Fluid consumption was then measured six hours later and the alcohol preference ratio calculated as described in Chapter Two. The measurements were taken six hours later as measured central effects of both RU38486 and spironolactone appear to fast acting and transient (Be Kloet, 1991).

ACTH $_{4\text{-}10}$ injections

Previously screened male low and high alcohol preferring mice (25-30g) received i.p injections of $ACTH_{4\text{-}10}$ (10 mg/kg) or saline vehicle (n=6 per group).

In addition two groups of previously screened low preferring mice (25-30g) received i.p injections of either 10mg/kg $ACTH_{4-10}$, 5mg/kg $ACTH_{4-10}$ or saline vehicle(n=8 per treatment).The mice were injected at 5p.m. and the preference was measured at 10 a.m. the next day. The low preferring groups also had their preference measured at 12, 24 and 36 hours after receiving injections. Preference was monitored for the following two days.

CRF antagonist

Previously screened low alcohol preferring mice (25-30g) received an i.c.v. injection of either α-helical CRF_{9-41} (Tocris Cookson Ltd, Bristol, U.K.) 5μg in 2μl, or saline vehicle (n=8 per group) at 4 p.m. Fluid consumption was measured at 16, 36 and 60 hours after the injection.

Statistical analysis

In the results data is expressed as mean ± SEM. A priori assumptions enabled comparison between treated and untreated groups in the corticosterone antagonist experiments to be performed using Students' t-test. The other experiments were analysed by two way analysis of variance followed by Dunnett's post hoc-test were appropriate.

Results

RU38486 and Spironolactone Drugs

Fluid consumption and mouse weight, in the high alcohol-preferring mice used, were not affected by the injection procedure. The effects of the administration of RU38486 are shown in figures 4.1a and 4.1b. Following the administration of RU38486 (150ng) there was no significant alteration in alcohol preference or total fluid consumption. Spironolactone administration also had no effect on alcohol preference (Figure 4.2a) or total fluid consumption (Figure 4.2b) when compared with vehicle administration alone.

Figure 4.1a

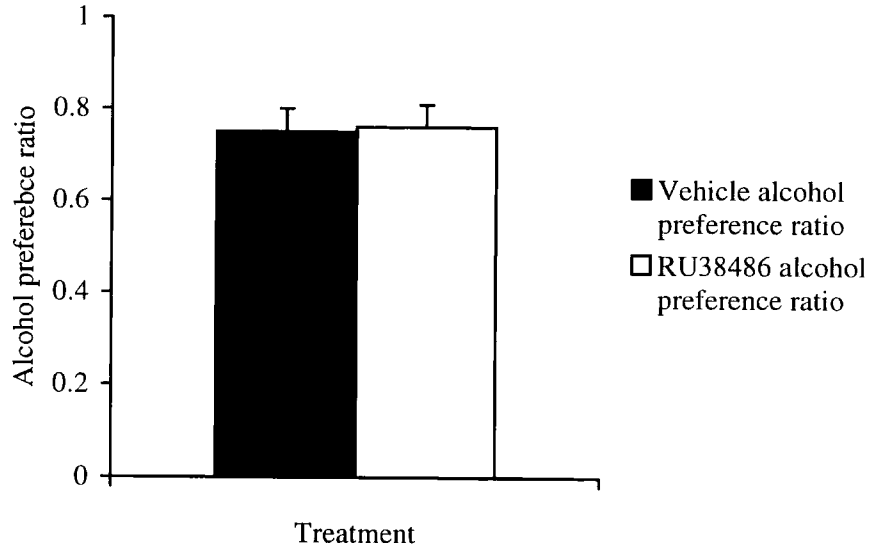


Figure 4.1b

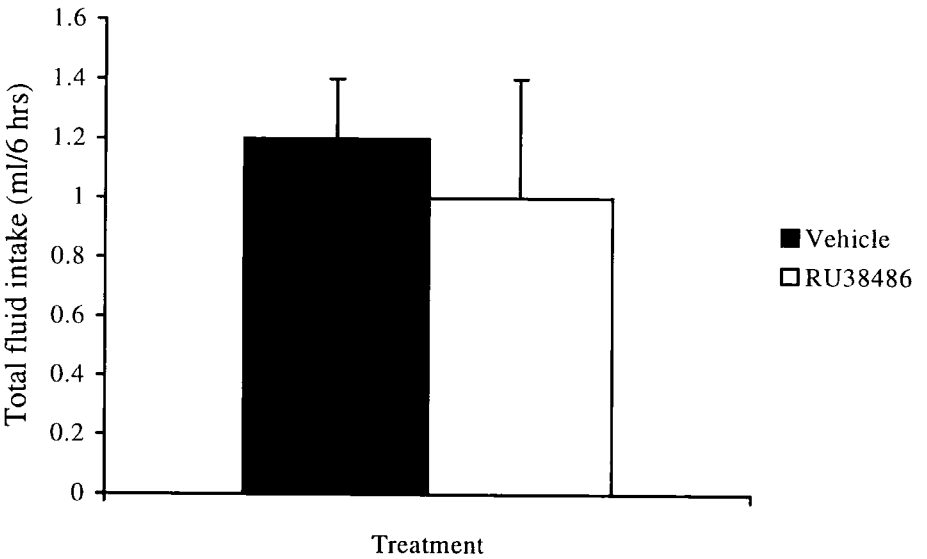


Figure 4.1. *Figure 4.1a shows the mean preference ratio ± sem of male C57 mice and Figure 4.1b shows the total fluid consumption of the same mice (n=8 per group) following i.c.v. injection of 150ng RU38486 or tween vehicle. Administration of the drug did not alter alcohol preference or total fluid consumption when measured six hours after the injection.*

Figure 4.2a

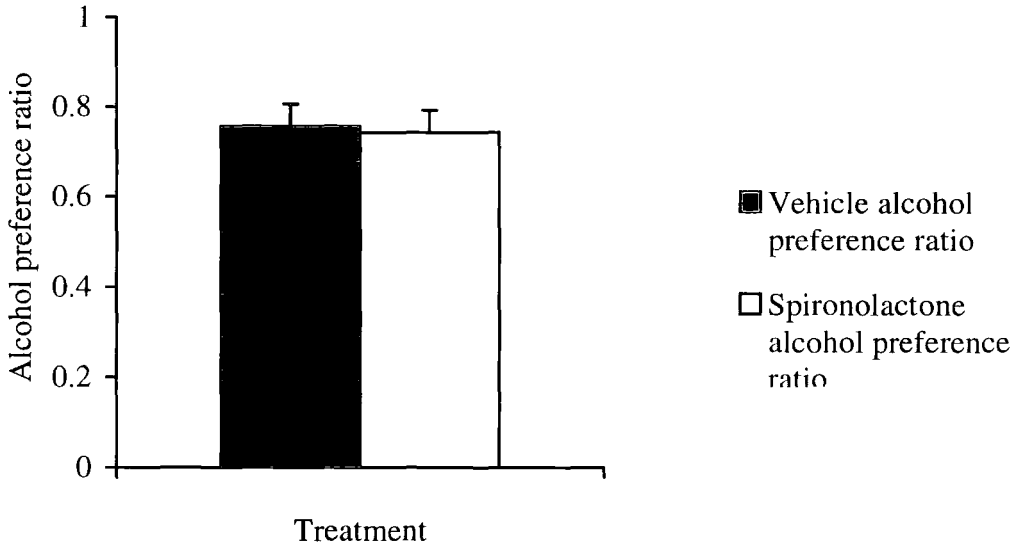


Figure 4.2b

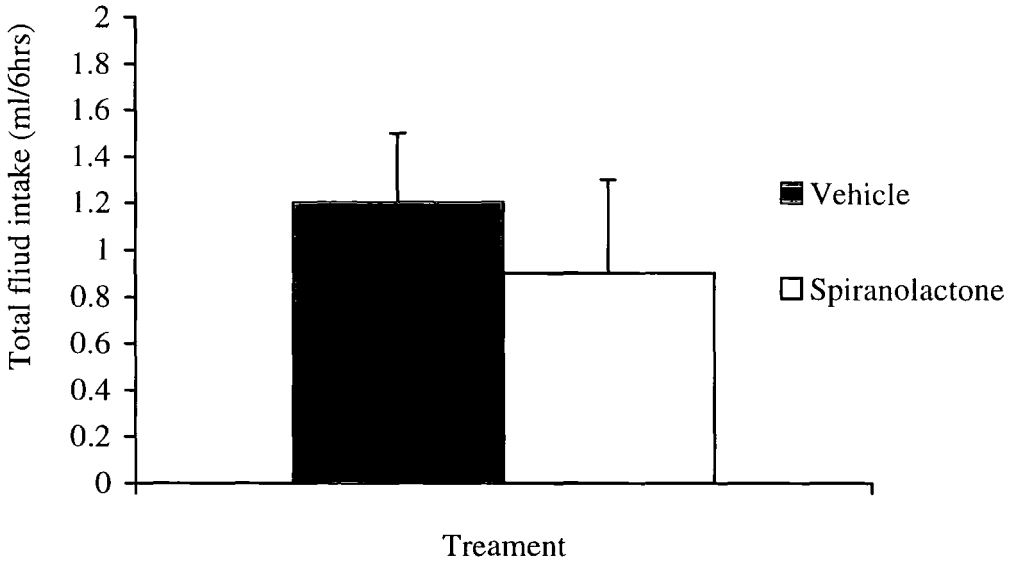


Figure 4.2. *Figure 4.2a shows the mean preference ratio ± sem of male C57 mice and Figure 4.2b shows the total fluid consumption of the same mice (n=8 per group) following i.c.v. injection of 150ng spironolactone or tween vehicle. Administration of the drug did not alter alcohol preference or total fluid consumption when measured six hours after the injection.*

Metyrapone injections

Administration of metyrapone in a daily single injection of 50mg/kg did not alter the alcohol preference compared to tween control (Figure 4.3). However when this drug was given twice daily at a dose of 100mg/kg it significantly reduced alcohol preference when measured during the treatment ($P<0.001$, for day 6, day 5, day 4 and day 3 of the treatment schedule compared with the corresponding vehicle injections Figure 4.4). At this dose no effect of the drug was observed on total fluid consumption (Figure 4.5). The body weights of the animals that received metyrapone injections were also unaltered (26 ± 1g both before and at the end of the treatment).

Figure 4.3a

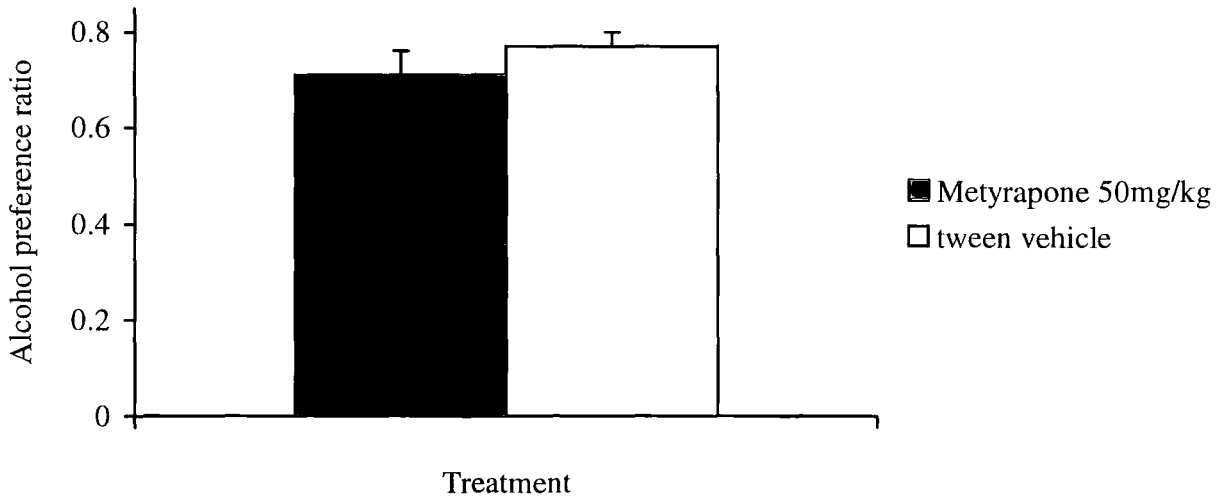


Figure 4.3b

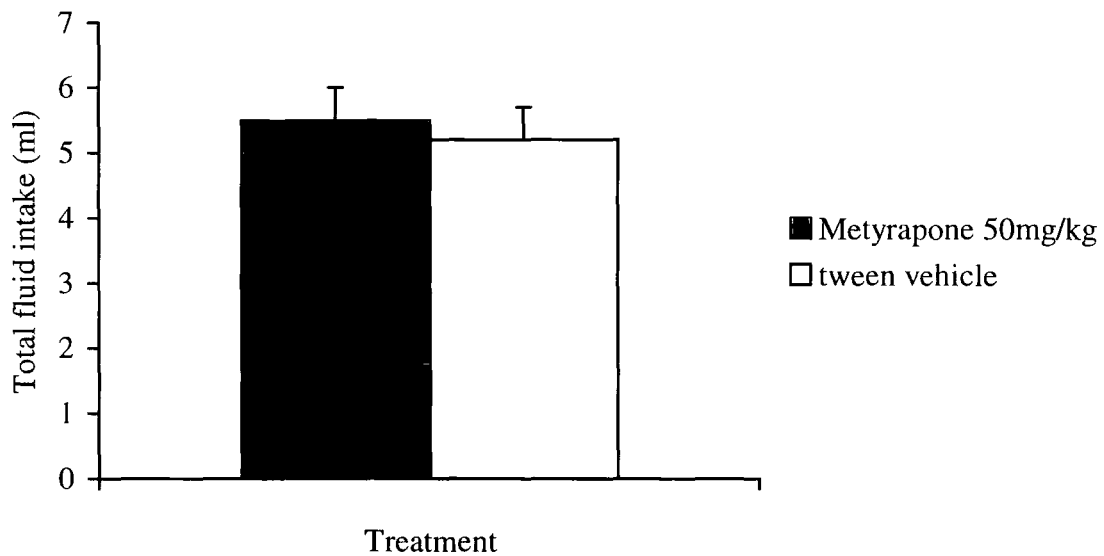


Figure 4.3 *Figure 4.3a shows the mean alcohol preference ratio +/- SEM of male high alcohol preferring C57 mice after an i.p injection of 50mg/kg of Metyrapone. This dose did not significantly alter alcohol preference. Figure 4.3b shows that total fluid consumption in the same mice was also unaltered by administration of metyrapone*

Figure 4.4

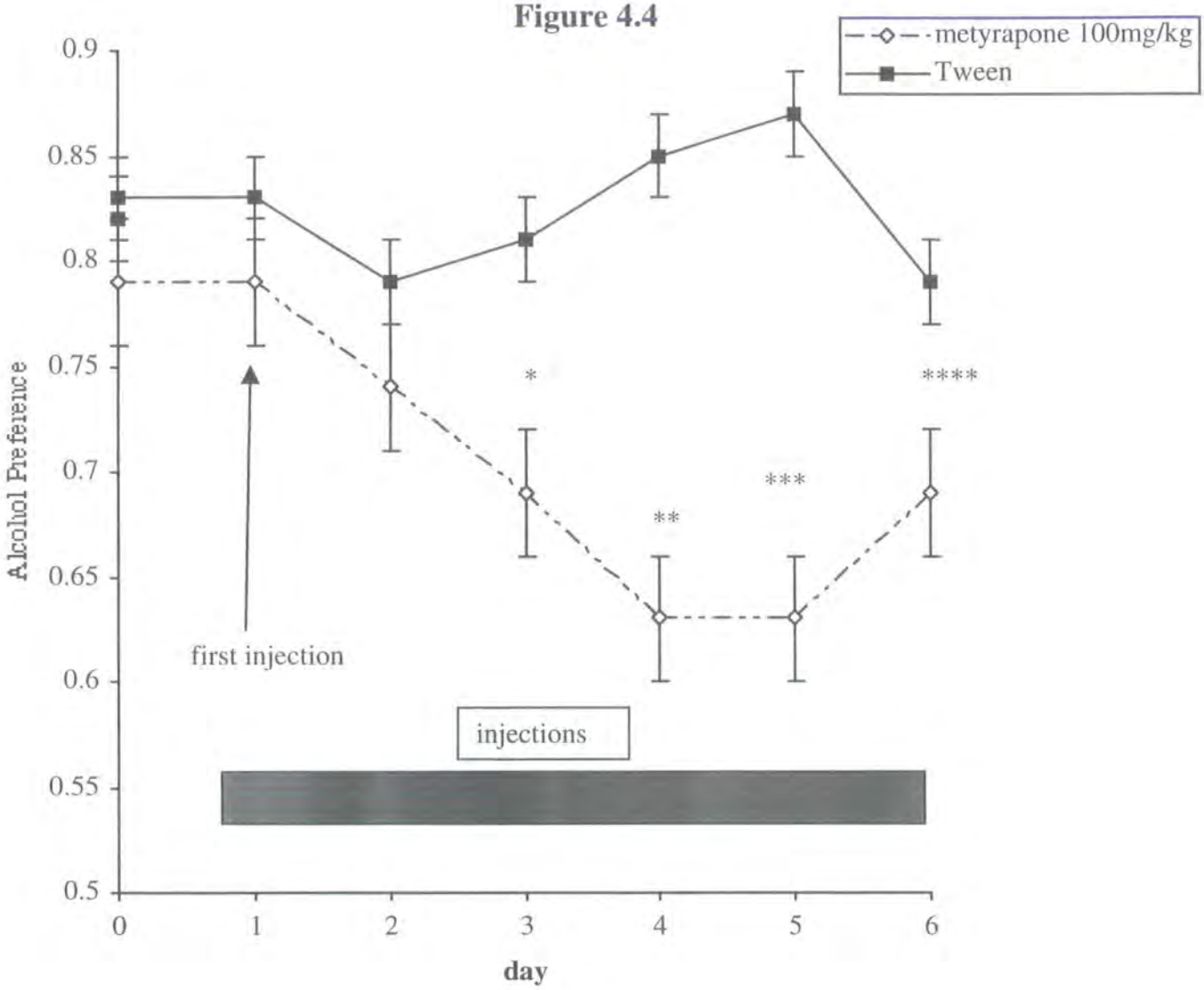


Figure 4.4 *The alcohol preference of C57 mice when either injected twice daily with metyrapone (100mg/kg) 0r tween vehicle. Metyrapone injections significantly reduced alcohol preference measured on days 3, 4, 5 and 6 compared with the both vehicle treated group and the pre-injection baseline measures. (****P<0.001 day 6 vs. vehicle; ***P<0.001 day 5 vs. vehicle; **P<0.001 day 4 vs. vehicle; *P<0.001 day 3 vs. vehicle),*

Figure 4.5

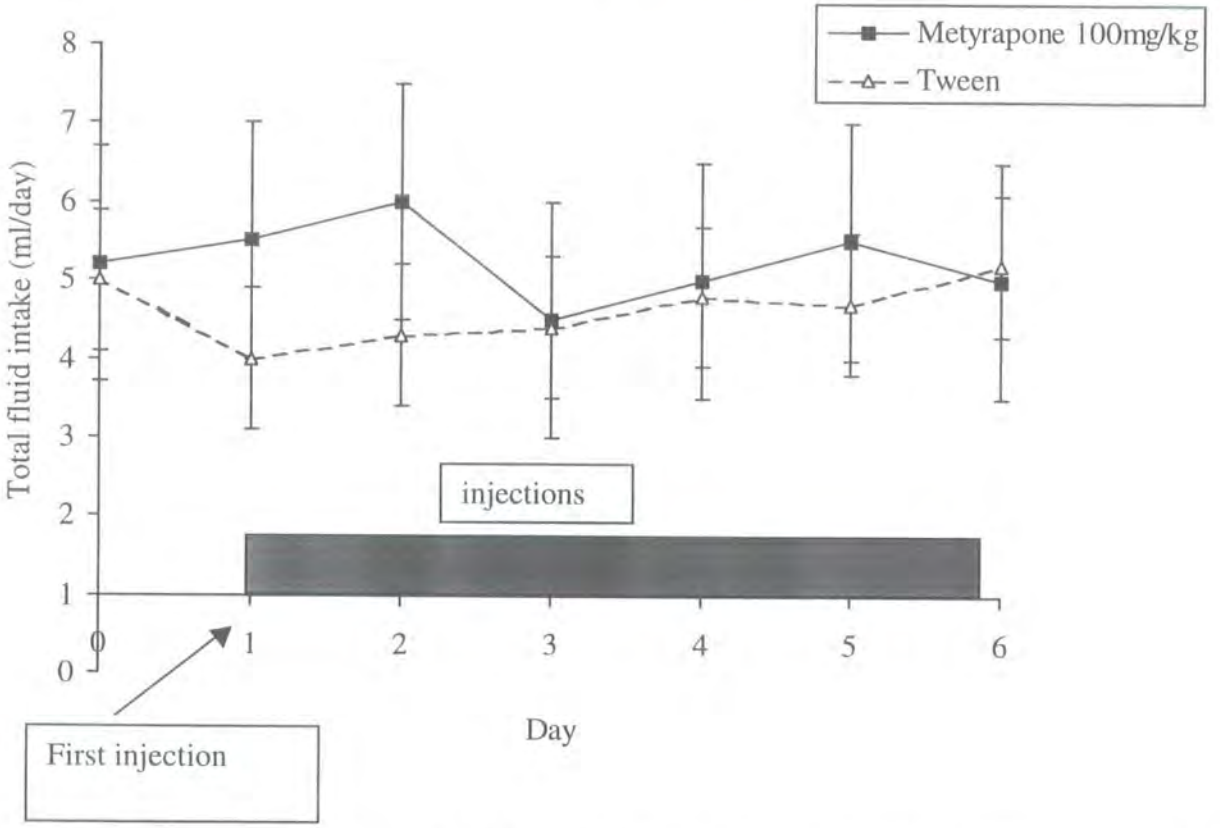


Figure 4.5 *The total fluid intake in high alcohol preferring C57 mice treated with either 100mg/kg metyrapone or vehicle injection. Injection schedule did not alter total fluid intake compared between treatments or to baseline values.*

ACTH $_{4\text{-}10}$ injections

Administration of the ACTH fragment ACTH $_{4\text{-}10}$ (10mg/kg) in high preferring mice decreased ($P<0.001$ vs. control and $P<0.001$ vs. baseline) alcohol preference, measured 12 hours after the injection was given (Figure 4.6). This effect was transient and after twenty four hours preference had returned to pre-treatment levels. The ACTH fragment did not alter fluid consumption.

However in low preferring animals injections of 5mg/kg and 10mg/kg $ACTH_{4\text{-}10}$ increased alcohol preference. Figure 4.7 shows 24 hours after the injection the larger dose of $ACTH_{4\text{-}10}$ increased alcohol preference ($F=5.6$ $P<0.01$) compared with baseline ($P<0.01$) and vehicle injected ($P<0.05$) animals. However, alcohol preference in the vehicle injected mice was increased 48 hours after the injection ($P<0.01$).

Injections of 5mg/kg $ACTH_{4\text{-}10}$ increased alcohol preference 36 hours after the injection ($F=3.6$ $P<0.05$) compared with both baseline values and the saline vehicle injected group ($P<0.05$). This increase was not seen at the earlier measurement time point, 12 hours. This data can be seen in Figure 4.8.

Figure 4.6

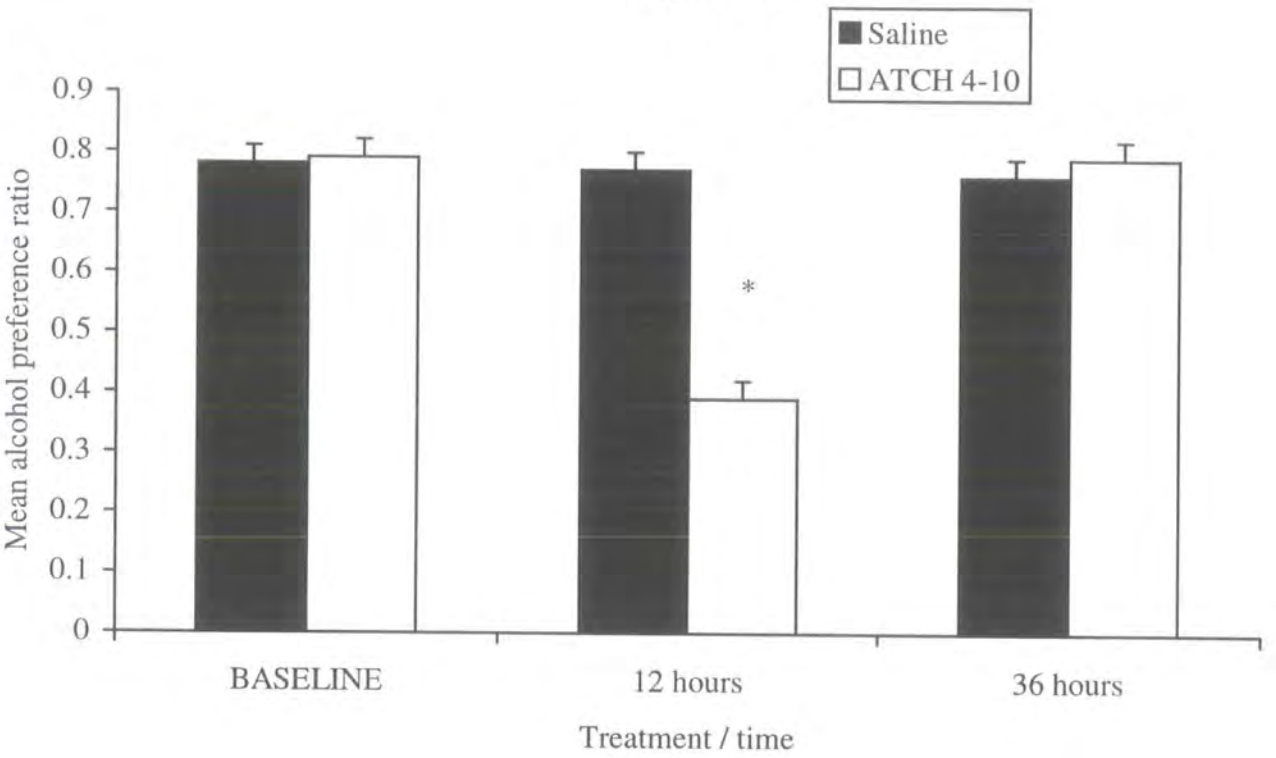


Figure 4.6 *Alcohol preference of mice (n=6 per group) that either received a single injection of ACTH (10mg/kg) or saline vehicle, preference was measured 12 hours and 36 hours after the injection (*p<0.001) $ACTH_{4\text{-}10}$ injected compared with both baseline and vehicle control).*

Figure 4.7

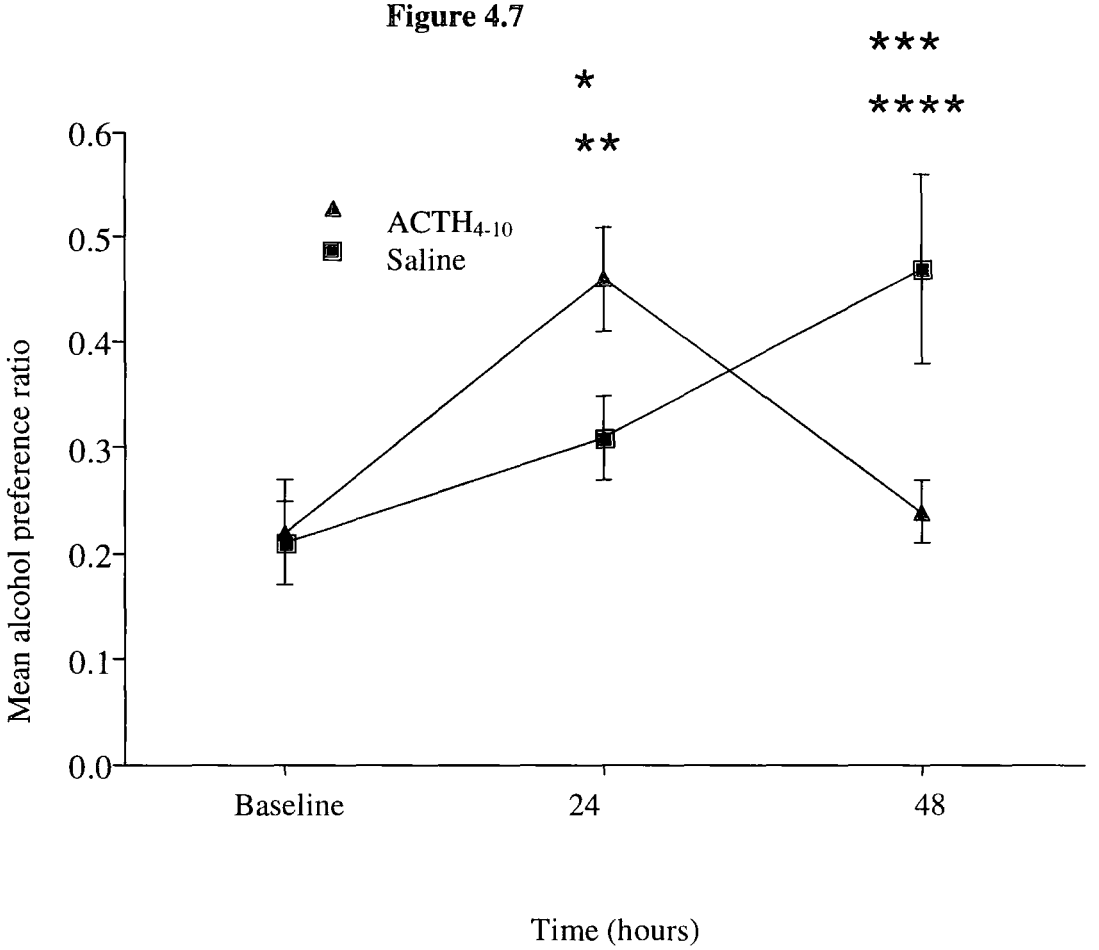


Figure 4.7 *Administration of 10 mg/kg ACTH $_{4-10}$ increased alcohol preference in low preferring mice, 24 hours after injection, compared with both baseline (*P<0.01) and vehicle injected controls (**P<0.05). 48 hours after the injection alcohol preference was increased in the vehicle injected group compared with both baseline (*** P<0.05) and ACTH injected mice.*

Figure 4.8

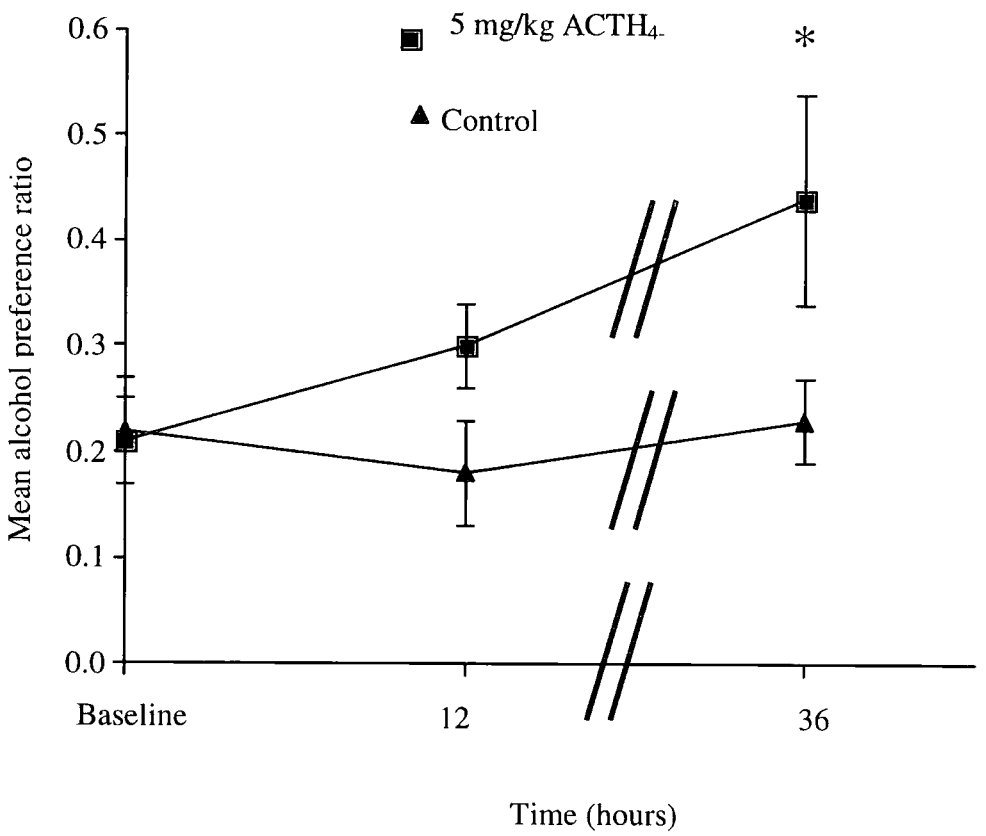


Figure 4.8 *Administration of 5mg/kg ACTH $_{4-10}$ increased alcohol preference in low preferring mice, 36 hours after injection, compared with both baseline and vehicle injected controls (**P<0.05).*

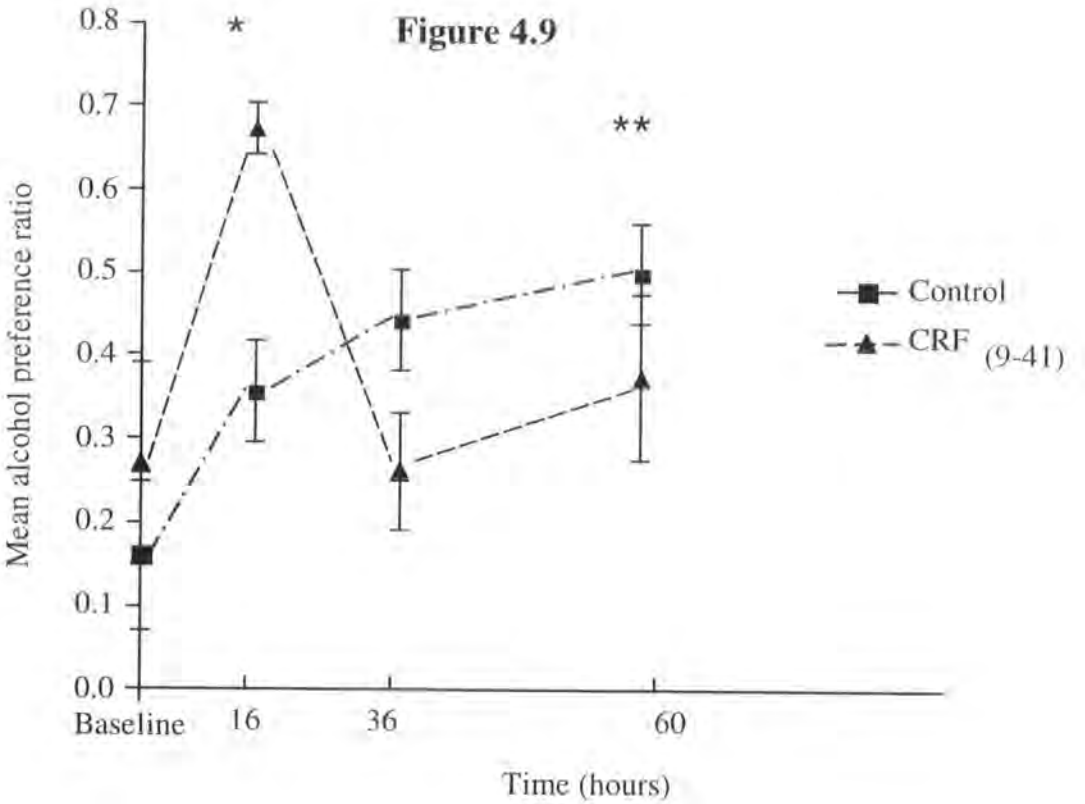


Figure 4.9 *Alcohol preference in low preferring C57 mice after a single injection of α-helical CRF_{9-41}. Administration of the CRF antagonist significantly increased alcohol preference compare to baseline and vehicle injected mice ($P<0.01$). Alcohol preference in vehicle injected mice increased 60 hours after the single injection **($P<0.01$).*

Discussion

Metyrapone injections

Fahkle et al., (1994a,b) have shown decreases in voluntary alcohol consumption in rats following adrenalectomy. This reduction was reversed by administration of 200mg of corticosterone, as a subcutaneous pellet, over 60 days. Fahkle and colleagues also demonstrated a reduction in alcohol consumption in rats when the formation of corticosterone is blocked using metyrapone. They report a reduction in intake after a single injection of 50mg/kg of metyrapone in the rat. In the present study a 50mg/kg injection had little effect on alcohol preference, but a twice daily injection of the larger dose (100mg/kg) was effective in reducing alcohol consumption. Another difference from the findings of the present study was that no change in total fluid consumption was seen; in the Fahkle study there was a small significant reduction in total fluid consumption. The reduction in alcohol intake caused by metyrapone is not likely to be due to accumulation of corticosterone precursors, as decreases in alcohol consumption in rats are precipitated by surgical adrenalectomy that reduces corticosterone levels, but without the accumulation of corticosterone precursors. A mechanism explaining the influence of corticosterone on alcohol consumption is not easy to find, and is complicated more by the results of the antagonist results.

Receptor antagonist administration

The animals did not appear to have an aversive reaction to the metyrapone, although no quantifiable measures were taken to assess the 'malaise' reported to be a side effect of the drug (Fahkle et al., 1994b). The lack of effect of both RU38486 and spironolactone on alcohol preference, given the

effect of suppression of corticosterone synthesis on alcohol consumption, is on first inspection surprising. However, previous reports have failed to demonstrate an effect of both centrally (Fahkle et al., 1999) and systemically (Fahkle et al., 1995) administered corticosterone antagonists on alcohol consumption.

To conclude that corticosterone type I and type II receptors are definitely not involved in corticosterone's modulation of alcohol intake may not be possible just on the evidence of this study or the previous studies on rats. It may be the case that the doses of the antagonists were not effective as they may have been too low. The doses of the drugs used had an anxiolytic effect when given i.c.v. in rats (De Kloet, 1991). They were also the maximum doses possible to be given using the apparatus for conscious i.c.v. administration. Both drugs are insoluble in water or other polar solvents and dissolving them in organic solvents might cause vehicle effects, so the method of suspending the drugs in dilute tween was used. Previous work (Watson, 1992) established that 0.05% tween has no adverse effects on mice when administered i.c.v. in a volume not exceeding 5μl. Higher doses of antagonist suspensions are beyond the limit of the current apparatus. It cannot be discounted that the drugs when administered I.C.V. as in this study, or systemically as in the rat studies, may in fact not be reaching their targets in sufficient concentration to be effective. Unpublished data from this lab (Seth, 1999) has shown that sub-cutaneously administered corticosterone failed to increase alcohol preference in C57 mice, and i.p injections of corticosterone have been ineffective in raising alcohol preference in low alcohol consuming rats (Fahkle et al., 1994a). Administration of corticosterone by subcutaneous pellets and by direct implantation in the brain (only when implanted into the ventral striatum, but not the hippocampus, septum, or thalamus) has both been effective

in increasing alcohol consumption (Fahkle & Eriksson, 2000). It may be possible that with direct implants into selected brain areas some effect of the antagonists may be seen.

Although the link between alcohol consumption and corticosterone is becoming well established, it does not necessarily hold that corticosterone type I or type II receptors are involved in the maintenance of self-administration or preference. In addition to the results obtained from the present study, Fahkle et al., (1994a; 1995) have shown that type I and type II agonists are equally ineffective in modulating alcohol intake when administered both systemically and centrally. The mechanism of corticosterone influence on preference could be mediated by another as yet unidentified corticosterone receptor; perhaps even the elusive membrane bound receptor that has been suggested by some researchers (Sze and Towle 1993, Chen et al., 1993; Orchinik, 1999). There is, however, no evidence that this 'receptor' does not bind RU38486. The evidence for this receptor is mainly based on the observations that corticosterone has rapid actions that, because of the speed are not genomic. RU38486 has been shown to inhibit the potentiation of excitatory amino acid responses in the VTA neurons (Cho & Little, 1999), this would suggest that rapid non-genomic actions if not mediated by the classical GR are mediated by another receptor that at which RU38486 acts as an antagonist. This would suggest that an alternative receptor for corticosterone is not candidate for the influence of corticosterone on alcohol consumption.

$ACTH_{4-10}$ and CRF injections

A single dose of $ACTH_{4-10}$ transiently reduced the alcohol preference of high preferring C57 mice. A similar effect has been demonstrated in rats

(Krishnan et al., 1991). However in low preferring mice both doses increased alcohol preference. This increase in preference fits with the observation that injections of ACTH (10mg/kg) increase the rates of lever pressing during extinction of food-reinforced behaviour (De Weid & Jolles, 1982) i.e. that ACTH promotes reward related behaviour.

There are a number of plausible pathways for explanation of the reduction of alcohol consumption seen in high alcohol preferring mice. An interesting observation is that a reduction or ablation of corticosterone levels enhances ACTH release (Dallman et al., 1992). This is a consequence of reduced corticosterone feedback on the hypothalamus and this lack of inhibitory feedback increases CRF and ACTH release. It remains an interesting possibility that the presence or absence of corticosterone may influence alcohol consumption not by a direct central mechanism, but indirectly through decreased negative feedback on hypothalamic and pituitary peptides.

However, the injections in low preferring mice increased alcohol preference. The effects of ACTH on alcohol consumption would appear to be dependent upon the behavioural (i.e. whether the animal is low or high alcohol preferring) state of animal. The mechanism of action of ACTH induced increases in alcohol consumption is not clear but the relationship between POMC and endogenous opioids would seem to be a likely candidate. Various lines of correlative and experimental evidence indicate that endogenous opioids play an important role in the reinforcing properties of alcohol. Alcohol preference in C57BL/6 mice correlates with hypothalamic β-endorphin levels alcohol is also reported to increase plasma β-endorphin levels (Gianoulakis et al., 1996), A correlation between increased β-endorphin and the risk of alcoholism in humans

has been proposed (Gianoulakis et al., 1996). POMC in addition to being the precursor of ACTH is also the precursor of β-endorphin. Administration of ACTH may interact with the endogenous opiate system thereby altering alcohol consumption.

Administration of the CRF antagonist increased alcohol preference in low preferring mice. These results appear to support previously reported observations that CRF decreases alcohol preference (Bell et al., 1998). This appears to confirm the influence of CRF on alcohol intake. Recent experiments in the laboratory (Croft, 2001) have shown that α-helical CRF antagonist had no effect on alcohol preference, or total fluid consumption in the high preferring mice.

Inhibition of corticosterone synthesis increases the levels of ACTH and CRF, because of reduced negative feedback. It is possible that high levels of both or either peptide in the absence of high levels of corticosterone have an inhibitory effect on alcohol consumption. This idea fits with the reduction in alcohol intake seen in the high preferring mice after ACTH administration and the increase seen in low preferring mice seen after CRF antagonist administration.

Vehicle induced increases in preference

Increases in alcohol preference were observed after administration of the i.c.v. saline, this increase may have been due to the stress of the injection procedure. It developed over time and so was not seen in the initial i.c.v. injection experiments performed using the corticosteroid receptor antagonists because measurement in those experiments did not continue after six hours. The

CRF antagonist may have blocked the development of this increase, possibly by an anxiolytic action (Baldwin et al., 1991).

An i.p injection of saline increased preference 48 hours after the injection. In Chapter Three the effects of single saline injections on alcohol preference were reported. It was found that single saline injections did not increase alcohol preference, however, this was measured only three weeks after the injection, not two days as in the present case. Increases in preference were not seen after a single saline injection in the experiments on the effect of CCK_B antagonists on alcohol intake in low preferring mice (Little et al., 1999). In these experiments preference was measured daily. A single injection of saline could increase alcohol intake, but this may have been an isolated case as in the other two studies it has not been seen.

Future work

The experiments using the corticosterone antagonist described in this thesis used a single (large dose) on high preferring mice. Repeated injections of both antagonists in low preferring mice could be undertaken to examine whether they would block the increase in alcohol preference seen after vehicle injections. More work using more doses and different ACTH fragments needs to be performed, in both high and low preferring mice to gain a better understanding of the influence of ACTH on alcohol consumption. An investigation into the factors that govern the availability of the different fragments could also provide valuable information about factors governing stress and alcohol dependence. Similarly a more complete examination of the effects of CRF and its antagonists, using more doses, in both high and low preferring mice needs to be performed.

Chapter Five

Blood and brain corticosterone levels

Introduction

Background

In Chapter Three increased alcohol preference after multiple saline injections was described. The injection procedure was assumed to activate the HPA, thereby increasing the circulating levels of corticosterone and decreasing levels of CRF and ACTH. In this chapter a series of measurements of corticosterone levels in response to saline injections, handling and baseline levels in stock mice were measured. As described in Chapter Two, circulating corticosterone is either bound to CBG or 'free'. Measurements of levels of both free and bound corticosterone were undertaken in order to define fully the corticosterone response to these manipulations.

The consensus has generally been that only non-protein bound (free) steroids are available for movement out of capillaries and into cells. There they either cause a biological response or are cleared from the circulation via a variety of metabolic pathways. It is reasonable to think that CBG acts as a buffer to provide a reservoir of corticosterone that is immediately available during times of stress, or at other times when activation of the HPA necessitates large increases in corticosterone. In stress CBG levels can rapidly decrease and so increase the amount of free or available corticosterone in the circulation (Brien, 1991).

However, it is also possible that the reduction in CBG is a mechanism of excretion of increased levels of corticosterone, as free corticosterone can be

excreted whereas bound corticosterone is not excreted. The question of what exactly a measure of free corticosterone is should be remembered. In addition to measurements of blood corticosterone levels, the brain levels of the hormone in selected areas (the cerebral cortex, hippocampus, and striatum) were also measured.

Despite the increasing interest in the central effects of corticosterone, only circulating levels of total corticosterone in the blood tend to be routinely measured. Other markers of HPA axis activity are also measured including ACTH, adrenaline, noradrenaline, CRF and CBG levels, but it is very infrequently that free and bound levels of corticosterone are measured. The circadian changes in corticosterone blood levels spanning the 24 hr photoperiod, and changes seen during and after stress (of almost every kind) have been reported extensively (Bulwalda et al., 1999; De Kloet et al., 1996). The effects of different behavioural and pharmacological manipulations on central corticosterone receptors have also been reported in numerous studies. The central effects of corticosterone are of interest to many researchers investigating a spectrum of physiological, pharmacological and endocrinological areas, yet none measure brain levels of corticosterone. Changes in Kd, Bmax, expression of mRNA of corticosterone receptors, and of metabolising enzymes are all of value in assessing adaptations of neurotransmitter and hormonal systems, but of equal importance, but ignored, are the levels of ligand in these systems. Investigators into stress, and the HPA axis responses to stress will measure other parameters of HPA activation e.g. ACTH levels and CBG levels. Measurement of brain corticosterone levels is not performed and seems to be ignored. Literature reports of central corticosterone levels appear to be confined to one paper in the mid

seventies that examined brain corticosterone levels in neonatal and perinatal rats (McEwen et al., 1974). The previous reports on brain corticosterone levels were performed without the advantage of the now commercially available RIA kits. However, these corticosterone RIA kits are normally used for the assessment of corticosterone levels in blood, saliva, or urine. It was, therefore necessary to develop a novel procedure to measure brain corticosterone levels.

Saline injections and corticosterone levels

The increase in alcohol preference caused by repeated saline injections develops slowly, as shown in the previous experiments; the increase is normally apparent after three weeks. In this study the effect of multiple saline and single injections on corticosterone levels was investigated. The effect of multiple injections was examined using previously identified low alcohol preferring C57 mice. Alcohol when given i.p (Ellis, 1966) or as liquid diet (Tabakoff et al 1978) has profound effects on the HPA axis, increasing corticosterone levels. In Chapter Three it was demonstrated that the injection-induced increase in alcohol consumption was not dependent on the availability of alcohol during the injection schedule. Therefore corticosterone levels could be measured after giving the injection procedure alone, thus avoiding the complication of changes in corticosterone levels caused by increased alcohol consumption. The effect of a single saline injection on blood and brain corticosterone levels was also examined; this was performed in naïve mice. Although the existence of two distinct sub-populations of alcohol preferring animals was demonstrated, there was no evidence for a biphasic distribution of total corticosterone levels (Chapter Two).

Corticosterone levels in the hippocampus were measured because it is an important target for the action of adrenal steroids. It expresses a high number of both glucocorticoid and mineralocorticoid receptors (DeKloet et al., 1996). Adrenal steroids modulate the excitability of hippocampal neurons and influence the magnitude of long-term potentiation (e.g. DeKloet et al., 1997). They also participate (along with excitatory amino acids) in regulating neurogenesis of dentate gyrus granule neurons and stress induced atrophy of the dendrites in CA3 (McEwen et al., 1993, 1995, 1998). The striatum (including the caudate, putamen and the nucleus accumbens in this study) was chosen because it has been shown both in vivo (Rouge-Pont et al., 1998) and in vitro (Rouge-Pont et al., 1999) that administration of corticosterone to this area increases dopamine release. This is reported to be the mechanism by which corticosterone influences reward related behaviour. Little work is performed on the effects of corticosterone in the cortex. This region was chosen to contrast with the hippocampus and striatum. Glucocorticoid receptors are also found in the cortex and so this region was also chosen for investigation.

Methods

Effects of single saline injections on blood and brain corticosterone levels

Group housed male C57 mice (25-30g) were from stock cages and either injected with saline (i.p 0.1ml/10g) (n=6), or handled but not injected (n=6). The procedure was performed at the midpoint of the light cycle (12-1 p.m.).The mice that were handled were removed from their home cage and held as if to receive an injection, but not injected, then placed back into the home cage. Ten minutes after the intervention the mice were removed from its home cage and taken to the a work bench outside the room and killed by cervical dislocation before blood and brain samples were taken (cortex, hippocampus & striatum) for corticosterone RIA. The mice were taken out of the room to be killed to avoid elevating the corticosterone levels of the other mice, vocalisation by the mouse being killed or the scent of blood may have induced rises in the corticosterone levels of the remaining mice. Fresh disposable gloves were used on each occasion.

In response to stress there is a rapid elevation of corticosterone measurable after 1-2 minutes; this increase subsides after about one hour (Tannenbaum et al., 1997). Ten minutes was, therefore, chosen as the time point for sampling as the time when peak or near to peak corticosterone levels might be measured in response to the injection stress.

An additional group of mice (n=6) was used, these mice were taken individually from the stock cages for immediate sampling. These samples were assayed after the ethanol extraction procedure detailed below.

Effects of repeated saline injections on blood and brain corticosterone levels in low preferring mice

Mice were identified as low alcohol preferring using the three week preference screening procedure (as described in Chapter Two). These mice (n=6 per group) were either only handled, or injected with saline, once daily (at 2-3 p.m.) for three weeks (21 days). On day 22 the mice were killed at the time when they would have been due to receive their injection or, for the other group, to be handled. These brain samples were assayed using the protocol detailed below.

Corticosterone assays

Total blood corticosterone levels

The RIA assay for blood corticosterone levels was performed as described in chapter two using the antibodies supplied by ICN.

Free corticosterone assay

The method used for measuring free corticosterone levels had been previously described (Meaney et al., 1992). In this method a known concentration of labelled corticosterone is incubated with a plasma sample. The labelled corticosterone will compete with endogenous bound corticosterone for CBG, so measuring the amount of added tracer bound will give the proportion of endogenous tracer that is bound; if the total levels are measured the free corticosterone levels can then be calculated.

20 µl of ^{3}H corticosterone tracer (stock ^{3}H cortciosterone with a specific activity of 79 Ci/mmol, diluted 1/1000 to give 10,000 cpm) was added to 50 µl of each plasma sample and incubated for 30 minutes at 37°C. The mixture was placed on ice for 10 minutes and 1 ml of dextran treated charcoal was added; this

was kept on ice for 5 minutes more before centrifugation. Triplicate samples of 200µl of the supernatant were added to a scintillation vial, 5ml of scintillation cocktail was added and then counted in a scintillation counter.

Free corticosterone calculation

% Free Corticosterone = {(cpm added-cpm counted)/cpm added} x 100

[Free Corticosterone] = % Free Corticosterone x [Total Corticosterone]

Preparation of brain areas for RIA

Two methods of preparation were used to measure the brain levels of corticosterone. The first involved a simple homogenisation step before RIA, the second involved ethanol extraction of the corticosterone from the brain sample before RIA. The ethanol extraction method was used to overcome the potential (although not obvious) problems of the dispersion of corticosterone in the suspension. It was theoretically possible that the tracer corticosterone may have bound to fragments of membrane lipids, or proteins. This may have resulted in artificially high measured brain corticosterone levels after the separation procedure in the final RIA.

Method one

Homogenate RIA

The brain samples were homogenised in four volumes distilled water (w/v). To 0.05ml aliquots of the resultant suspensions 0.45ml of Tris-HCl buffer was added. The standard radioimmunoassay procedure was then performed on these samples.

Tracer recovery

Although method one appeared to produce reliable results, a test to investigate the distribution of corticosterone in the homogenate was performed. Although it was assumed that the endogenous corticosterone and added tracer would be evenly dispersed in the homogenate and they would be freely available to bind with the antibody, this could not be certain. To investigate this 20µl of tracer (10,000 cpm) was added to brain samples, then 1 ml of either distilled water (n=6) or 100% ethanol was added (n=6). These samples were then homogenized and centrifuged 20 minutes 6000 xg. The supernatants were decanted into scintillation vials and the radioactivity counted in a liquid scintillation counter. The percentage of recovery of tracer was then calculated.

Method two.

Alcohol extraction of corticosterone.

The brain regions for the mice were homogenised in x4 volumes of 100% ethanol and then centrifuged for 20 minutes at 13,000xg. The supernatants were then decanted into test tubes and placed in a drying oven. When the ethanol had evaporated the sediment was first dissolved in 10ml 100% ethanol before the addition of 1.5 ml standard RIA buffer (Tris-HCl, NaCl, NaN_3, BSA). The

samples were then assayed for corticosterone using the ICN antibodies and the standard protocol.

Statistical analysis

The results are expressed as mean ± SEM. One way analysis of variance was used to analyse the affects of the different treatments on blood and brain corticosterone levels. Significant interactions were followed by analysis of the main effects and if appropriate Fisher's pots hoc test.

Results

The recovery of tracer from the samples homogenised in 100% ethanol was over 90%. Those samples that were homogenised in water had a recovery level of below 78%. These results are illustrated in Figure 5.1.

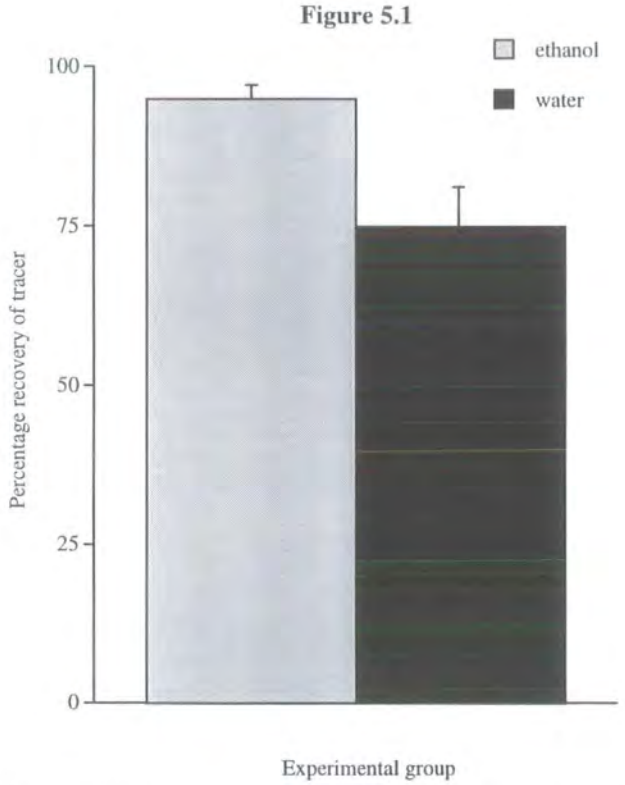


Figure 5.1 *The percentage recovery of ^{3}H corticosterone after homogenisation and centrifugation in either 100% ethanol or distilled water. Recovery of tracer after ethanol was greater than 90%.*

Corticosterone levels after a single injection

For clarity the results of the brain level measurements are expressed in graphical form firstly according to treatment and then according to brain region.

Corticosterone levels in the brain regions of stock mice

The corticosterone levels significantly varied between the different brain regions with treatment groups ($F_{2,45} = 3$ $P<0.001$). Post-hoc analysis in stock mice showed hippocampal levels of corticosterone were significantly higher than both cortical ($P<0.05$) and striatal ($P<0.001$) levels. However there was no significant difference between striatal and cortical levels (Figure 5.2).

Corticosterone levels in the brain regions of handled mice

In the mice that were handled without being injected, hippocampal levels were significantly higher than both cortical ($P<0.001$) and striatal ($P<0.001$) levels. Cortical levels were higher than striatal ($P<0.01$) (Figure 5.3).

Corticosterone levels in the brain regions of injected mice

Saline injected mice had significantly higher hippocampal than cortical ($P<0.05$) and striatal levels ($P<0.05$). There was no significant difference in cortical and striatal (Figure 5.4).

Corticosterone levels in the hippocampus of all treatment groups

Hippocampal levels were significantly different in the three groups ($F_{2,15} = 7.28$ $P<0.05$). Post-hoc analysis showed significantly higher levels in injected mice compared to handled ($P<0.05$) and stock ($P<0.001$). There was no difference between stock and handled mice (Figure 5.5).

Corticosterone levels in the cortex and striatum of all treatment groups

There was no difference in corticosterone levels in the cortex of the groups (Figure 5.6). Striatal levels, however, were significantly higher ($F_{2,15}$

=176 P<0.0001) in the injected mice compared to both the stock (P<0.001) and the handled mice (P<0.001). Handled mice had significantly lower levels than stock mice (P<0.05) (Figure 5.7).

Circulating levels of corticosterone

Analysis of variance showed total circulating levels of corticosterone in the different treatment groups were significantly different ($F_{2,15}$ = 4.5 P<0.05), post-hoc analysis revealed elevated levels in the mice that received saline injections compared with the stock mice P<0.01). (Figure 5.8). Levels of free corticosterone were significantly higher in the injected mice ($F_{2,15}$ =10.4 P<0.001) compared with both handled (P<0.005) and stock (P<0.001) mice (Figure 5.9).

Figure 5.2
Stock mice

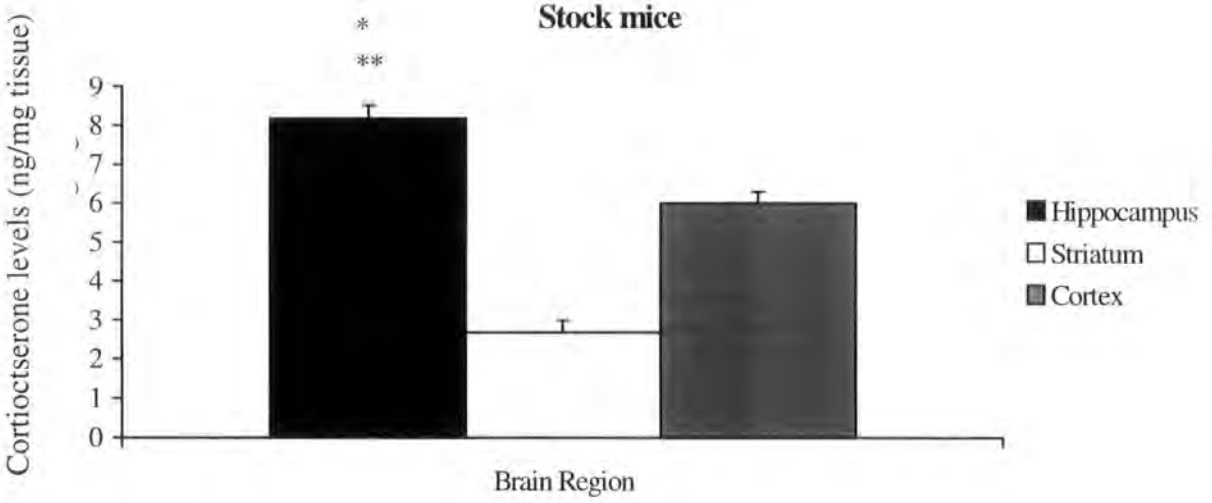


Figure 5.2 *corticosterone levels in the hippocampus, cortex and striatum of stock C57 mice. Hippocampal levels were higher than striatal (*P<0.001) and cortical (**P<0.05)*

Figure 5.3
Handled

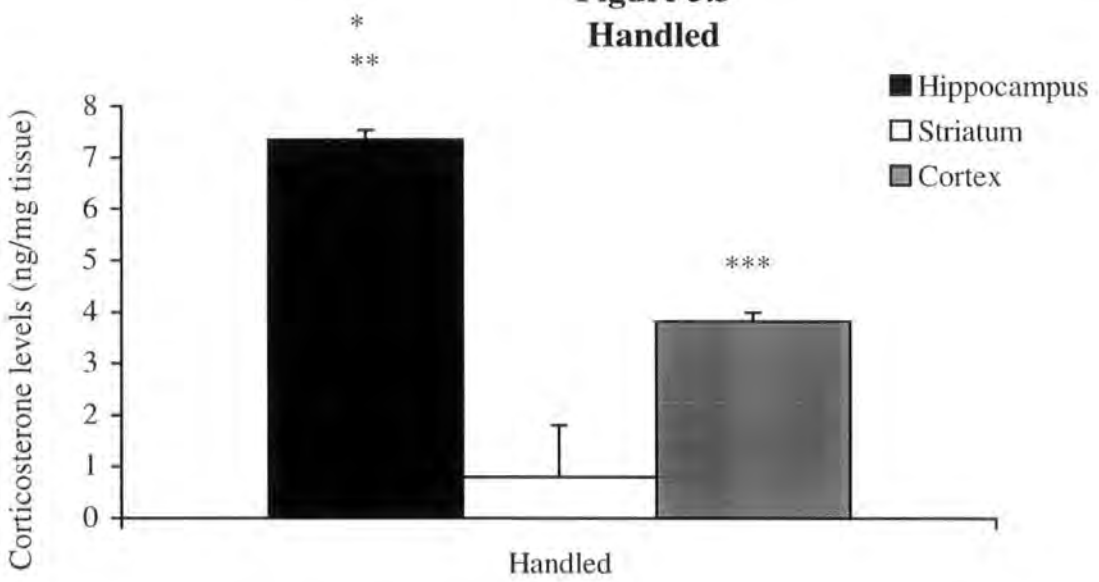


Figure 5.3 *Corticosterone levels in the hippocampus, cortex and striatum of handled C57 mice. Hippocampal levels were higher than striatal (*P>0.001) and cortical (**P<0.001). Cortical levels were higher than striatal levels (***P>0.01).*

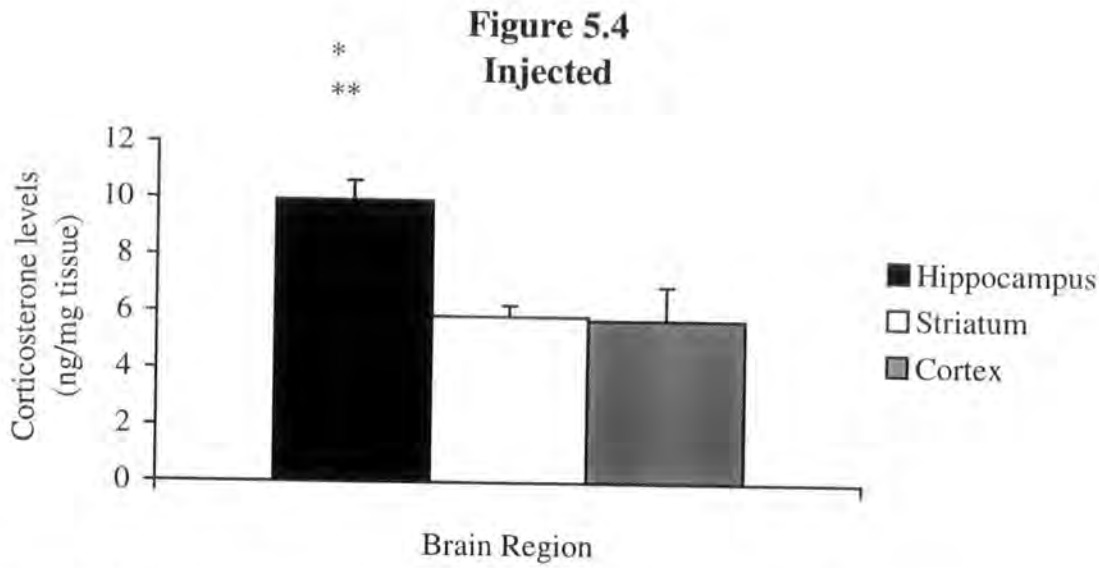


Figure 5.4 *Corticosterone levels in the hippocampus, cortex and striatum of injected C57 mice. Hippocampal levels were higher than striatal (*P>0.001) and cortical (**P<0.05).*

Figure 5.5
Hippocampus

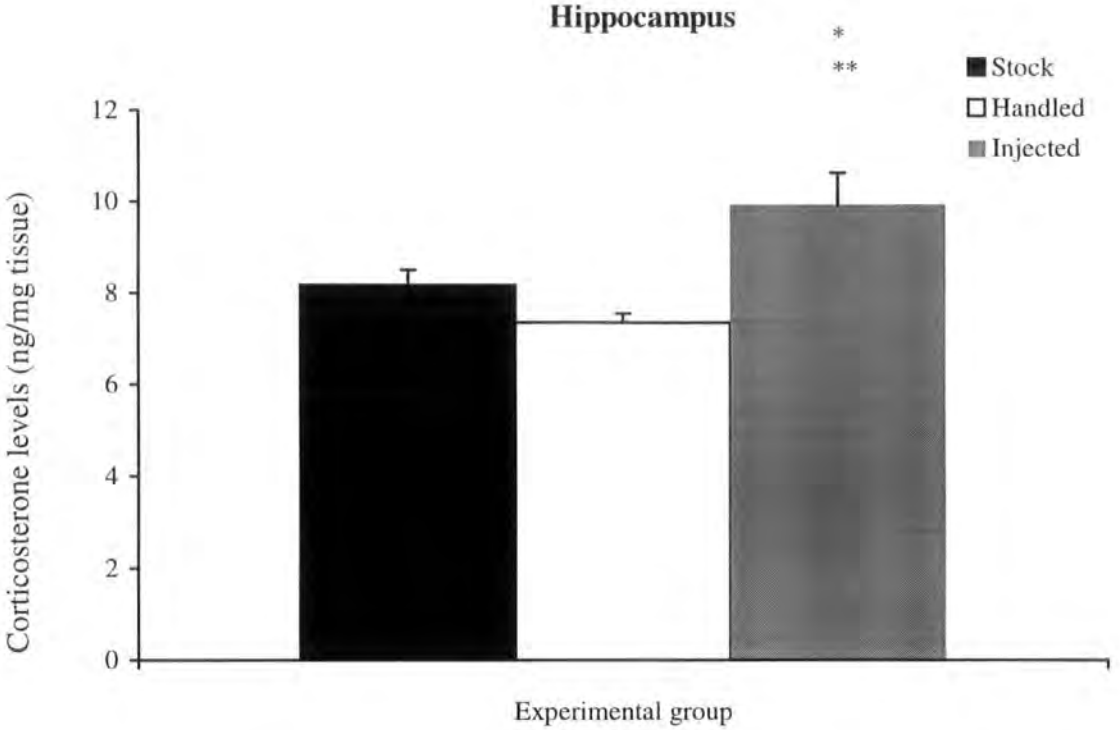


Figure 5.5 *corticosterone levels in the hippocampus of stock, handled and injected mice. Injected mice had higher corticosterone levels than both stock (*P<0.05) and handled mice (**P<0.001).*

Figure 5.6
Cortex

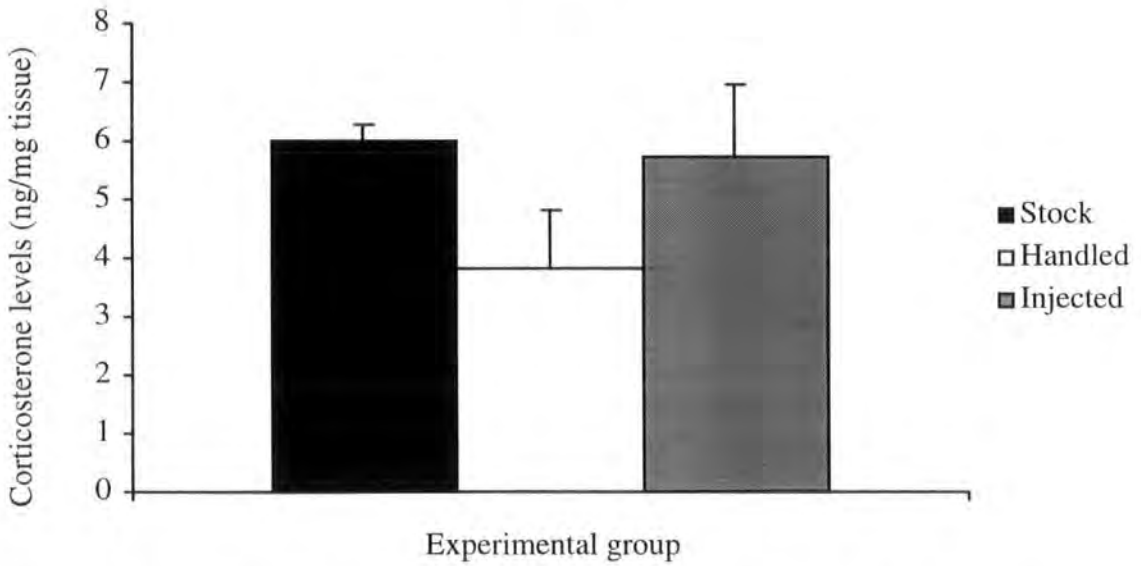


Figure 5.6 *No difference in cortical corticosterone levels between the treatment groups.*

Figure 5.7
Striatum

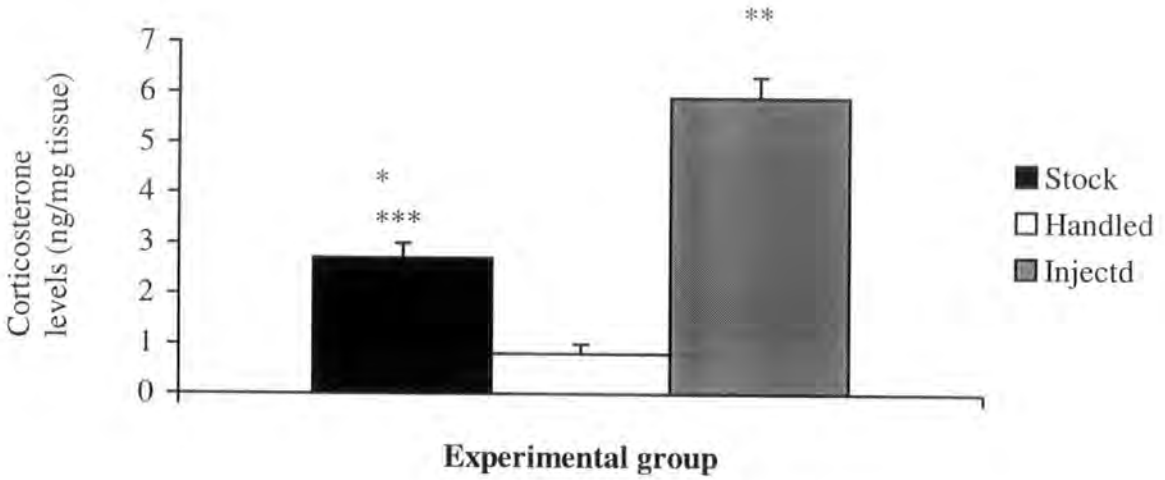


Figure 5.7 *Increased corticosterone levels in the striatum of injected mice compared to both handled (*P<0.05) and stock mice (**P<0.05). Handled mice had lower levels of corticosterone than stock mice (***P<0.05).*

Figure 5.8

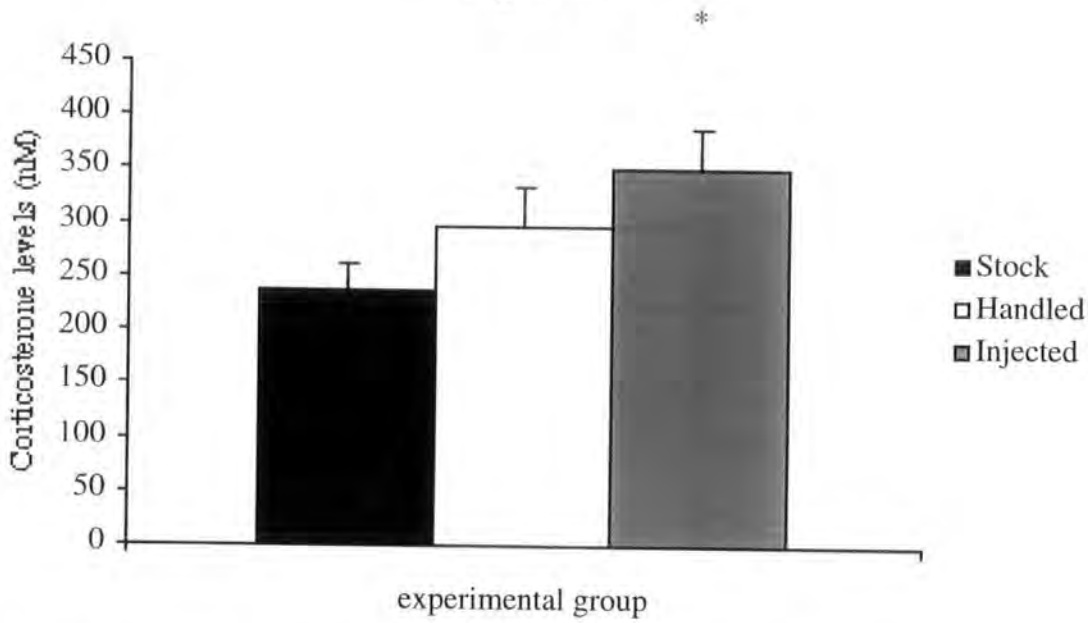


Figure 5.8 *Total blood corticosterone levels. Saline injections significantly (*P<0.01) increased corticosterone levels compared to the stock mice, but not to those that were handled.*

Figure 5.9

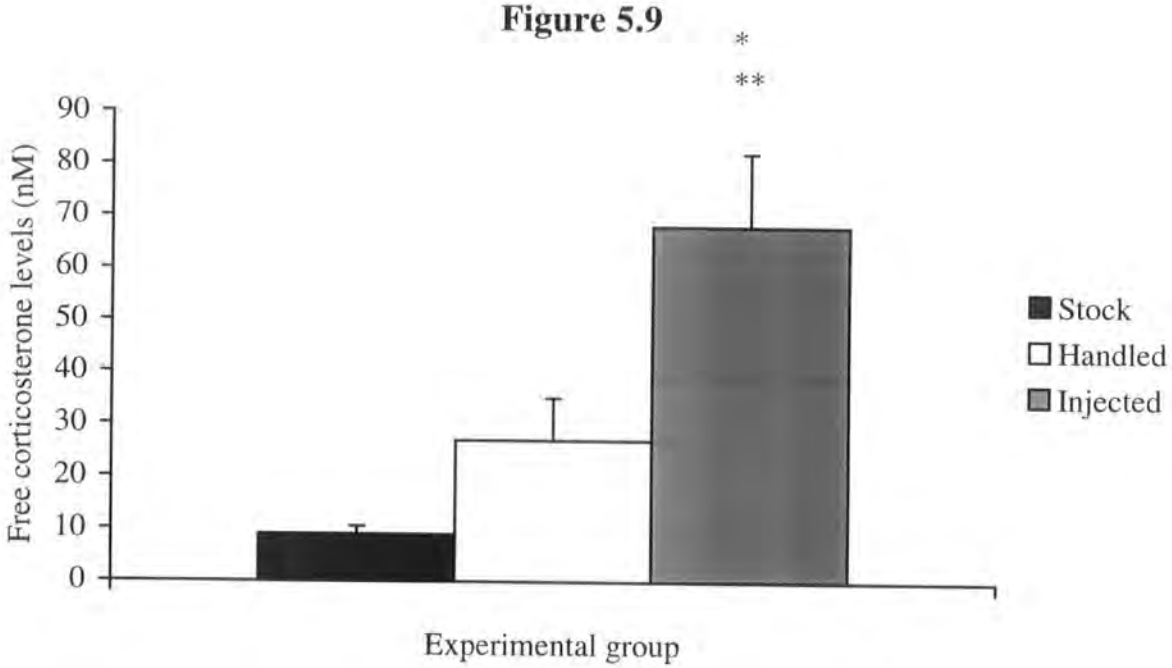


Figure 5.9 *Free blood corticosterone levels. A single saline injection significantly increased corticosterone levels compared to the stock mice (*P<0.005) and handled mice (**P<0.001).*

Repeated saline injections

Total Blood corticosterone levels showed no change between those handled and those injected (Figure 5.10). The blood levels measured were comparable to the levels measured in the mice that received a single saline injection in the previous experiments.

There was no difference in hippocampal corticosterone levels between the two treatment groups. Cortical levels also did not differ between treatments (5.11). However, corticosterone levels varied significantly in the two brain regions ($F_{1,11}$=10.64, P=0.0039).

Figure 5.10

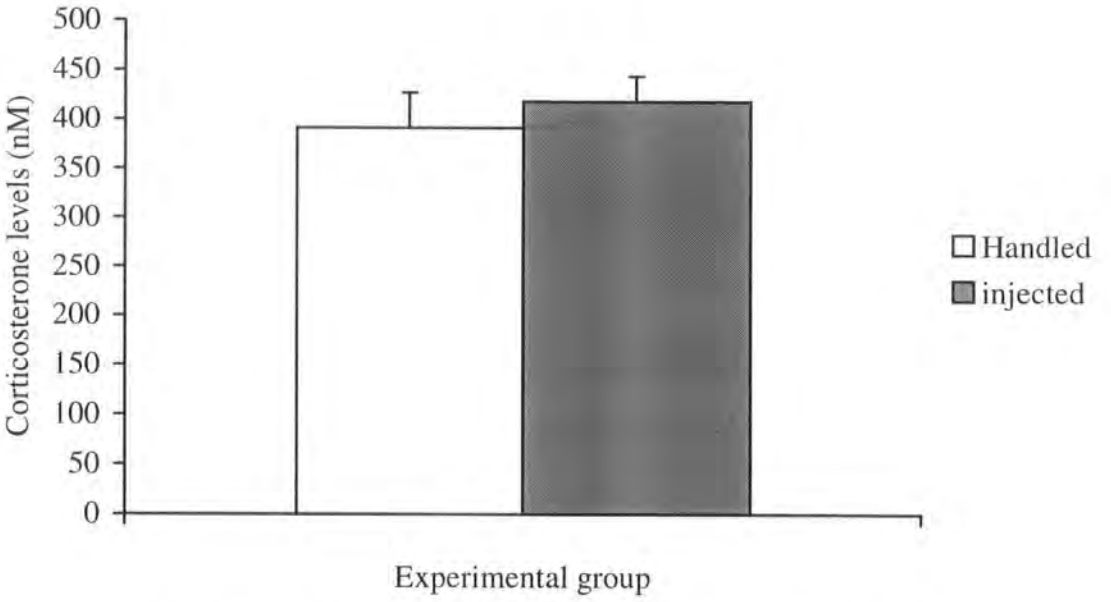


Figure 5.10 *No difference in total blood corticosterone levels between mice that received three weeks of daily saline injections and those that received three weeks of handling .*

Figure 5.11

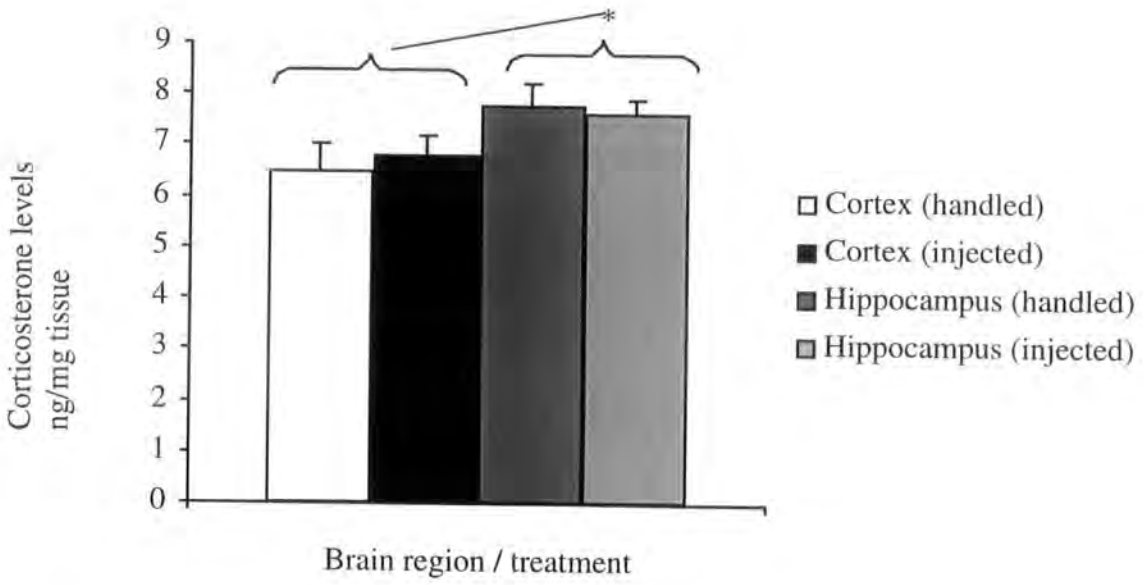


Figure 5.11 *No difference in cortical or hippocampal corticosterone levels between injected and handled mice, after three weeks of either daily saline injections or handling.. Hippocampal corticosterone levels were higher than cortical corticosterone levels irrespective of the treatment (*$F_{1,11} = 10.64$, $P<0.005$).*

Discussion

Brain corticosterone levels

Ethanol extraction produced a reliable, high level of recovery of labelled corticosterone when the procedure was tested for efficiency. Ethanol extraction was used as corticosterone is an organic insoluble (in polar solvents) compound, which will preferentially partition into an organic solvent. Although other similar molecules (other steroids) could be extracted at the same time the use of RIA using specific corticosterone antibodies means that contamination of the sample did not present a problem. The differential partition of corticosterone (as described in the methods section of this chapter) when the brain sample was suspended in water may have produced artificially high corticosterone measurements so the ethanol extraction method was adopted as the method of choice. Comparison of the levels measured in both preparations used would argue against this being a problem. However, it could not be guaranteed that this was not the case and so ethanol extraction became the preferred method.

There are, at least two, potential criticisms that could be levelled at each procedure. The first is the problem of corticosterone degradation by an endogenous enzyme, 11 β-hydroxysteroid dehydrogenase (Sekl, 1997). This enzyme catalyses the conversion of the active glucocorticoids, corticosterone and cortisol, to inert 11 keto-products (11-dehydrocorticosterone, cortisone), thus regulating access of glucocorticoids to receptors. The enzyme shows bi-directional activity in tissue homogenates and microsomal preparations, but may predominantly function as an 11β-reductase. Extraction of the corticosterone in 100% ethanol should make the impact of this enzyme negligible, but it should be a factor considered when refining the assay method further. One potential

solution would be to use inhibitors of this enzyme such as licorice or its derivatives (Sekl, 1997).

The second criticism that needs attention is the possibility of corticosterone being detected that originates from the cerebral circulation rather than the brain itself. The levels of corticosterone from this potential artifact are thought to be minimal, but are a factor that needs careful consideration when refining the assay. In the method now used in the laboratory, mice are transcardially perfused with saline in order to exsanginate and so avoid the potential for circulatory artifacts.

Brain corticosterone levels after single injection or repeated saline injections

In all the treatments; stock, handled and injected, the corticosterone levels in the hippocampus were the highest. The mice that received saline injections had the highest hippocampal corticosterone levels. The hippocampal formation is an important brain structure in episodic, declarative, and spatial learning and memory. It also exerts control over ACTH secretion and so is important in the functioning of the HPA axis (Vale et al., 1981). Hippocampal neurons express receptors for corticosterone (McEwen et al., 1974). The two receptors mediate a variety of effects on neuronal excitability, neurochemistry and structural plasticity (DeKloet et al., 1996). Increased corticosterone levels in the hippocampus could, therefore, exert an influence on behaviour and could provide a possible mechanism for the increased alcohol preference seen after repeated saline injections. However, when the levels of corticosterone were measured in the hippocampus after three weeks of saline injections there was no difference between the saline injected hippocampal levels and the handled hippocampal

levels. This observation could be explained by an adaptive response to the repetitive injection stress, resulting in a reduced corticosterone secretion after multiple injections compared to the increased levels recorded after the a single injection.

Corticosterone levels in the striatum of injected animals were higher than those of handled and stock animals. Corticosterone can have profound effects on the mesolimbic dopaminergic system – the reward pathway. In the VTA, corticosterone has been shown to potentate excitatory amino acid induced neuronal activity (Cho & Little, 1999). Corticosterone has been shown to increase dopamine output in the nucleus accumbens (Rouge-Pont et al., 1998; Piazza et al., 1993). Suppression of glucocorticoid secretion has opposite effects (Piazza et al., 1994). During stress, a selective block of stress-induced release of corticosterone reduces dopamine release by 50% (Piazza et al., 1994). The development of long-lasting sensitisation of the dopaminergic response to psychostimulants and opioids is suppressed by the blockade of corticosterone secretion. The increases in corticosterone levels in the stratum in the saline injected mice for the above reasons could present a plausible mechanism for the increased alcohol preference seen after repeated saline injections.

An unexpected finding was the higher levels of corticosterone in the striatum of the stock mice when compared to those of the handled mice even though the levels of free (potentially available) corticosterone were higher in the handled mice. This result suggests that circulating corticosterone cannot be an absolutely reliable guide to central availability. These results suggest that the mechanism that allows the entry of corticosterone into specific brain regions must be more than a passive process. If the entry of corticosterone into the

specific regions of the brain occurred as a result of passive diffusion the levels of corticosterone measured in the different brains regions would be similar and the levels would relate directly to the circulating levels of free corticosterone or even total corticosterone. This is not the case therefore an active mechanism would seem a likely explanation. The densities and availability of corticosterone receptors could also explain the differences in the levels of corticosterone between the different brain regions. The hippocampus has a the highest density of both MRs and GRs these receptors, whereas in the cortex and striatum there are relatively fewer receptors. These receptors could act as a reservoir for the circulating corticosterone and so explain the regional differences in the levels.

The effects of repeated saline injections on blood and brain corticosterone levels showed no changes in levels between the two groups. Only total blood corticosterone levels were measured in this experiment as the method for measuring the free levels had not been established in the laboratory at the time. There were differences in the corticosterone levels in brain regions measured, but not between the treatment groups. This is in contrast to the acute studies that showed differences between the treatments, this might be explained by an adaptive response to repeated stress. The lack of difference between the two groups led to the addition of the extract control (stock) group in the acute studies. Although, it should be noted that the striatal levels were not measured in this study, due to lack of time.

Future work

The development of an assay to measure brain corticosterone levels presents a massive opportunity for future work. Any experiment previously performed on stress reactions, manipulations of the HPA, chronic or acute stress that has been

previously performed that measured circulating levels of corticosterone could benefit with the additional information of measuring brain corticosterone levels. A literature search on the Webb of Science data base with the search words corticosterone levels (1984-present) produces thousands of reports of a vast array of different experiments all of which reported circulating levels of corticosterone as part of the experiment and none of which reported brain levels. A comprehensive list of the potential for future work could run into volumes even before totally novel ideas are considered.

Section Two

Previously alcohol preference and the factors modulating that preference have been examined in C57 strain mice. In this section the following group of experiments (Chapter Six and Seven) investigate the effects of alcohol administration and abstinence on TO mice. The experiments were performed after the acute withdrawal phase had passed in order to examine possible changes in the HPA axis (Chapter Six) dopaminergic receptors (Chapter Seven) that might aid the understanding of the phenomenon of relapse.

The experiments were performed after a 23 day chronic alcohol treatment (administered via a liquid diet) and a 6 day period of abstinence. The alcohol administration schedule was chosen as this produced changes in locomotor activity responses to psychostimulants develop after six days abstinence, whereas a shorter 10 day treatment did not (Manley & Little , 1997).

Long-term changes in neuronal and behaviour responses have been reported during abstinence at 24 hours, 6 days and 2 months of withdrawal (Bailey et al., 1997; Manley & Little, 1997). The following studies were intended to be performed at all these three time points, but time constraints meant that only the 6 day period was examined, except in the locomotor activity experiments in Chapter six where additional experiments were performed at 24 hours post withdrawal.

Chapter Six

Corticosterone and abstinence

Introduction

Corticosterone and dependence

As mentioned previously the HPA axis has an important influence on drug and alcohol dependence related behaviour. This chapter examined potential long-term changes in the levels and actions of corticosterone after chronic alcohol administration followed by a period of abstinence.

Studies of drug self-administration, in particular psychostimulant drugs, have shown that physical and psychological stressors facilitate the reinstatement of drug taking even after prolonged periods of withdrawal; in addition to facilitating the initial acquisition (Shaham and Stewart, 1995; Piazza et al., 1993). The relationship between stress and relapse in alcohol dependence is less well defined but a connection is becoming clearer. Amongst abstinent alcoholics, severe and chronic life stressors may lead to alcohol relapse. Brown and colleagues 1990) studied a group of men who completed inpatient alcoholism treatment and later experienced severe and prolonged psychosocial stress prior to, and independent of, any alcohol use. The researchers found that subjects who relapsed experienced twice as much severe stress before their return to drinking as those who remained abstinent (Brown et al, 1990).

Relapse is one of the major problems in the treatment of alcoholics. Alcoholics attempting to stop drinking will frequently go through the acute withdrawal syndrome and remain abstinent for weeks or months, but then relapse back to excessive and uncontrolled drinking. Prolonged effects of

excessive alcohol consumption, including craving and anxiety are seen in alcoholics after months or even years of abstinence. Rates of relapse amongst recovering alcoholics are very high. In alcoholics 1-2 years following therapeutic treatment rates of relapse of 74-90% (McKenna et al., 1993) and 80% (Naranjo & Kadlec, 1991) have been reported.

Acute administration of ethanol causes a dose dependent rise in plasma corticosterone levels in rats (Ellis, 1966). This adrenal response is dependent upon an increase in ACTH hormone secretion. Dexamethasone blocks the alcohol induced rise in plasma steroid (Adinoff et al., 1991). The influence of alcohol on corticosterone levels is considered to be exerted in a large part through the release of CRF. Removal of endogenous CRF or blockade of CRF receptors has been shown to reduce significantly the alcohol induced response of ACTH and corticosterone in rats (Rivier et al., 1996).

The effect of chronic alcohol administration on the HPA axis is not well defined. Alcohol is reported to alter the levels of plasma corticosterone but this is dependent on the method of alcohol administration even if the blood alcohol concentrations achieved are similar (Koranyi et al., 1987). Both persistent activation (Ellis, 1966; Tabakoff et al., 1978), or tolerance (Spencer and McKwen, 1990), of the HPA axis activity following ethanol administration has been shown. Chronic alcohol intoxication also leads to the disturbance of the normal diurnal variation of plasma glucocorticoids in rodents and in humans (Adinoff et al., 1991).

Persistent alterations in corticosterone levels are believed to produce adaptive responses in glucocorticoid receptor function. Stressful life events are believed to play an important role in the etiology of mood disorders (Anisman &

Zacharko, 1982; Shrout et al., 1989). Stress, even short lasting, can have profound and long lasting effects that may persist or develop after the stressor is no longer present (Post, 1992). These changes include persistent reduction in plasma CBG levels (Buwalda et al., 1999). The activation of the HPA by alcohol can be regarded in physiological terms at least as stressful. Whether alcohol ingestion is perceived as stressful is another matter, the tension reduction hypothesis would seem to suggest the contrary. This hypothesis suggests that drinking alcohol is performed to reduce stress rather than to promote stress; the validity of that hypothesis must be questioned in light of the evidence linking physiological stress reactions and alcohol consumption.

An investigation into whether alcohol induced changes in corticosterone levels could produce a lasting effect on anxiety related behaviour was performed. In the following experiments mice were administered alcohol for 21 days. After this the mice were placed on the elevated plus maze and the effects of the glucocorticoid receptor antagonist RU38486 on maze behaviours were investigated. The next set of experiments were designed to investigate the effects of corticosterone on locomotor activity following long-term withdrawal from alcohol treatment. Finally the levels of free, total and brain region corticosterone levels were measured to investigate whether changes in blood corticosterone levels reported during alcohol administration persist or develop further during long-term withdrawal. Long-term withdrawal is the term used in this work to describe a period of abstinence after chronic alcohol treatment (enough to produce physical dependence) that outlasts the acute withdrawal hyperexcitability. The importance of this was first discussed in the introduction.

The plus maze

The elevated plus maze paradigm is currently one of the most widely used animal models in behavioural pharmacology. The test is based on the aversion of rodents for open spaces and is reported to be bi-directionally sensitive to manipulations designed to influence anxiety. The efficiency of the test is dependent on a number of factors. If the strain of mice (or rats) has a low anxiety baseline, anxiolytic effects may prove hard to detect, conversely high anxiety baselines make anxiogenic effects hard to detect. Ethological measures were taken as well as the spatiotemporal measures to improve test sensitivity (Cole & Rodgers, 1995).

In this study an examination of the effects of i.c.v. injections of the GR antagonist RU38486 on mice behaviour on the elevated plus maze following six days of withdrawal from chronic alcohol treatment was undertaken. The purpose of this study was to investigate the possibility of long term changes in corticosterone receptor activity after alcohol treatment.

Glucocorticoids act on a variety of homeostatic processes including the behavioural expressions of fear and anxiety. Administered corticosterone, at levels designed to mimic stress-induced levels, increased anxiety related behaviour in a time-dependent fashion in the black/white box induced anxiety test. The most effective treatment time was 5 minutes after injection (Smythe et al., 1997). The plus maze and the social interaction test have demonstrated anxiolytic effects of corticosterone (Korte et al., 1996). Korte and colleagues have demonstrated an anxiolytic effect of both MR (spironolactone, dose of 150ng) and GR antagonists (RU 38486, dose of 150ng) in the elevated plus maze after exposure to a conditioned stressor. RU38486 (150ng) has been shown to

increase the time spent in the open arms of the plus maze when exposure was preceded by exposure to an environmental stressor (Korte et al., 1996).

Locomotor activity

The assessment of motor activity, first encountered in this Thesis in Chapter 2, is a common behavioural test used generally to define sedative and stimulant actions of drugs. Measuring the effects of corticosterone on locomotor activity in alcohol treated mice was chosen as a simple test for changes in the effects of corticosterone.

Classically steroid hormones receptors exert their effects acting as ligand-dependent transcription factors. Non-genomic (rapid) effects of corticosterone have been reported extensively in the literature. In rats, injections of corticosterone have been shown to have rapid stimulatory effects on locomotor activity. The administration of corticosterone produced a transient increase in locomotion in a novel environment an effect seen within seven and a half minutes that disappeared within 60 minutes of drug administration (Sandi et al., 1996). Rapid effects of corticosterone are best defined in male *Taricha* (newts) (Orchinik et al., 1998) and have also been described in white-crowned sparrows (Breuner et al., 1997). The assessment of the effects of corticosterone on locomotor activity in this study were divided into two basic experiments. The first was an investigation of the effects of corticosterone and the glucocorticoid receptor antagonist RU 38486 on locomotor activity in alcohol naive (stock) mice. The doses of corticosterone used (2.5-20mg/kg) were taken from a previous study that demonstrated an increase in locomotor activity after corticosterone injection in rats (Sandi et al., 1996). The second was an investigation of the effects of corticosterone on activity in alcohol treated mice at

two time points (i) twenty-four hours after the removal of alcohol and (ii) six days after the removal of alcohol.

Corticosterone levels

The elevated plus maze test and locomotor activity monitoring described above were designed to investigate possible behaviourally detectable changes in corticosterone receptor characteristics following alcohol administration. The effect of alcohol treatment and long-term withdrawal on the levels and availability of ligand (corticosterone) could provide important information that may help explain the effects of stress on relapse. Earlier in this chapter studies that have examined total corticosterone levels during alcohol administration or at intervals just after administration were described. There is no information regarding the circulating levels of total or free corticosterone at times after the acute withdrawal phase. The same is true for brain corticosterone levels.
In rats following stress, activity within the HPA axis is reported to subside rapidly and total corticosterone levels are normally indistinguishable from pre-stress levels as little as 1 hour. However, other measures of HPA axis activity such as CBG binding have been shown to still be increased in rats 24 hours after a single exposure to restraint stress (Tannenbaum et al., 1997). The levels of CBG are linked to the availability of corticosterone, so in this study the levels of free as well as total corticosterone were measured, also the corticosterone levels in the cerebral cortex, hippocampus and striatum were measured. These brain region were chosen for the reasons outlined in Chapter Five.

Methods

Drugs Used

Aldosterone, a MR agonist, corticosterone a mixed GR and MR agonist (Baxter & Rousseau, 1979) and RU 38486 a specific GR antagonist (DeKloet, 1991).

Animals

Adult male TO mice 14-18 weeks (26-40g, bred in the Psychology Department, Durham University), were housed in groups of ten (cage size: 45 x 28 x 13 cm). They were maintained in a temperature-controlled environment (21±°C) under reverse light/dark phase (lights on 2000 hours, lights off 0800 hours). The mice were maintained under reverse light/dark phase so that the behavioural testing and the measurement of corticosterone levels could be performed during their active phase (i.e. the dark phase).

Administration of alcohol

Alcohol was administered by a 23 day liquid diet (Lieber & Di Carli, 1986) schedule, previous described by Manley and Little (1997). In the administration all mice received a control diet for an initial period of three days. The mice undergoing ethanol treatment were then given a diet containing 3.5% ethanol for two days, followed by a 5% containing diet for nine days and finally an 8% diet for the last nine days of the treatment. Control groups were pair-fed iso-calorifically match control diet. There was no significant differences between the weights of the control and ethanol groups at the end of the treatment. The mean daily intake was 21.5 ± 2 g/kg/day.

Part 1

The elevated plus maze

Twenty minutes before testing each mouse (control or ethanol diet) received an intracerebroventricular injection (2μl) of either tween vehicle , 50ng, 100ng, or 150ng of RU38486 (*n* values of 7-8 per treatment group) The i.c.v injection procedure was described in Chapter Four. On the test day the mice were removed from the holding room and moved to the test room and habituated to that room for one hour prior to the start of the tests.

Apparatus (the elevated plus maze)

The elevated plus maze was a modification of the apparatus validated for NIH Swiss mice by Lister (1987). Two open arms (30 x 5 x 15 cm) and two closed arms (30 x 5 x 15 cm) extended from a common central platform (5 x 5), the entire apparatus was elevated to a height of 45cm above floor level. The maze floor was made from black Plexiglas, whilst the side and end walls were of clear Plexiglas. The open arms had a slight lip (0.25cm) to help prevent animals falling from the maze. All testing was conducted under dim red light (2x60W).

All testing was conducted during the mid-portion of the dark phase of the light/dark cycle. The mice were transported to the test laboratory at least one hour prior to testing. Testing commenced with the placement of a subject on the central platform of the maze facing an open arm. The mice were tested twenty minutes after receiving an injection, this has been demonstrated as the optimum time for the anxiolytic action of RU 38486 on the plus maze (Korte et al., 1996).

A standard five minute test duration was employed, and the maze was thoroughly cleaned between tests using damp and dry cloths. The maze exposure sessions were recorded using a video camera at a 45° angle to the maze. The camera was linked to a monitor and video-recorder in an adjacent laboratory. Video tapes were later scored blind to treatment conditions, using the Hindsight program.

Behaviours recorded were: number of closed and open arm entries (arm entry was defined as all four paws moved onto an arm), number of rears, and time spent on various sections of the maze including the central platform. These data were used to calculate the percentage open entries (open entries / total entries x 100) and the time percentage time spent in open and closed arms. In addition to these measurements, percentage time on the central platform; rearing frequency, the frequency of discrete behaviours such as head dipping (exploratory movement of head/shoulders over the sides of the maze), stretch-attend postures (SAP; an exploratory posture in which the mouse stretches forward and retracts to the original position without forward motion) were recorded. Head dips and stretch attend postures were differentiated as unprotected (on or from the open arms) or protected (on or from the central platform or closed arms).

Part 2 locomotor activity

(i) Stock mice

Adult male TO mice 14-18 weeks (26-40g) maintained under normal light/dark phase (lights on 0800 hours, lights off 2000 hours) were tested in the locomotor activity meters in the holding rooms. corticosterone (20mg/kg), RU 38486 (20mg/kg), corticosterone plus RU 38486 combination, both at 20mg/kg or dilute tween vehicle (0.05% tween in normal saline), for each treatment n=11.

A second experiment was also performed using the same basic procedure except that the mice were given aldosterone injections (1,2 and 4 mg/kg n=14 per group). Injections were given and the mice placed in the activity meters immediately after injection and their activity monitored for 30 minutes. The time from injection to activity measurement was minimal as the locomotor activating affects of corticosterone are rapid in onset and are transient. The experiments were performed between 10 a.m. And 4 p.m. (i.e. during the lights on).

(ii) Liquid diet treated mice

Mice received either alcohol liquid diet or control liquid diet (schedule described earlier in the methods section). When the diet schedule was complete the mice were then given standard laboratory chow and left undisturbed until testing. One group of mice (control and alcohol) were tested 24 hours after the removal of the liquid diet. The other group (control and alcohol) were tested six days after the removal of the liquid diet.

Activity testing

For the 24 hour and six day experiments the mice were tested in the holding rooms. Groups of control and diet mice were given either tween vehicle, 2.5mg/kg, 5mg/kg, 10 mg/kg or 20 mg/kg corticosterone injections (n=6-9 per injection group). Activity was monitored after a ten minute habituation period to the activity boxes. The experiments were performed from 10 a.m.- 4 p.m. (i.e. during the dark phase).

Part 3 Corticosterone levels

Corticosterone assays

The mice were treated with the liquid diet using the schedule previously described. When the diet schedule was complete the mice were then given

standard laboratory chow and left undisturbed for six days then the mice (n=6 per group) were killed and trunk blood samples collected into heparinized tubes for the total and free corticosterone assays. The brains were removed and the cerebral cortex, hippocampus and striatum dissected out over ice, the samples were then rapidly frozen until required for the assay. The total corticosterone levels were measured using the ICN antibodies and procedure described in chapter two. The proportion of free corticosterone and hence total corticosterone levels were assayed using the methods described in chapter five. The brain corticosterone levels were measured by RIA after ethanol extraction.

Statistical analysis

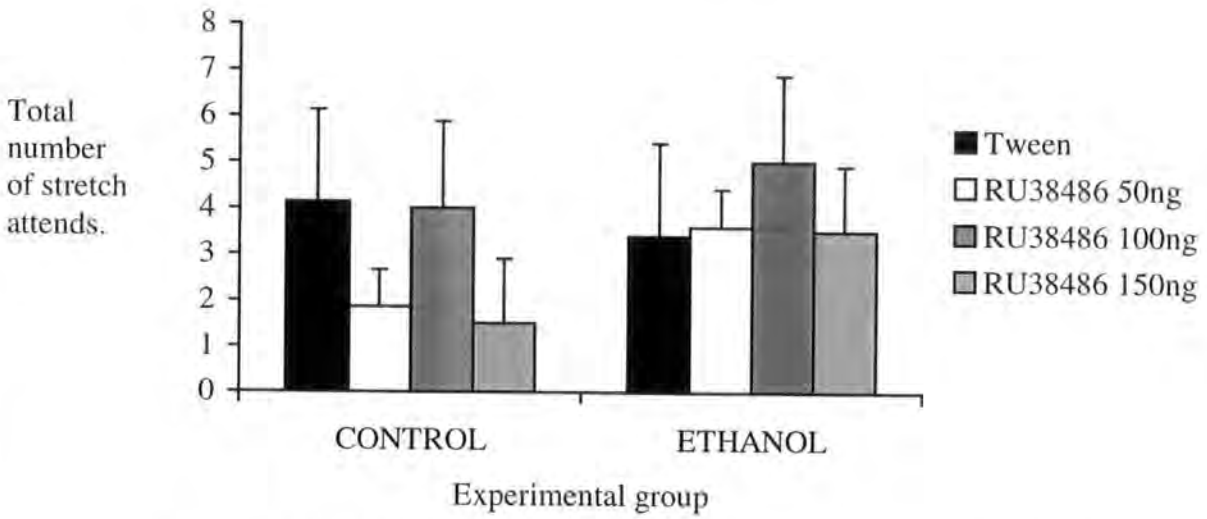
The locomotor data and the plus maze data was analysed by one way analysis of variance. When indicated, further comparisons between groups were performed using a Fishers' post-hoc test. Significance was set at $P<0.05$. Comparison between the two experimental groups, in the corticosterone levels experiments, were performed using Student's t-test.

Results

Plus maze

Analysis of open arm stretch attends (Figure 6.1), open arm head dips (Figure 6.2), central square rears (Figure 6.3), central square stretch attends (Figure 6.4), central square head dips (Figure 6.5), closed arm rears (Figure 6.6), closed arm stretch attends (Figure 6.7), closed arm entries (Figure 6.8) and open arm entries (Figure 6.9) showed no significant changes in any of the behaviours between diet treatment and/or drug administration.

Figure 6.1



Figures 6.1 – 6.12 *Behaviour on the elevated plus maze in male TO mice that receive alcohol or control liquid diet followed by six days abstinence and then i.c.v. injections of RU38486.* **Figure 6.1** *No significant effect of diet or drug treatment on open arm stretch attends*

Figure 6.2

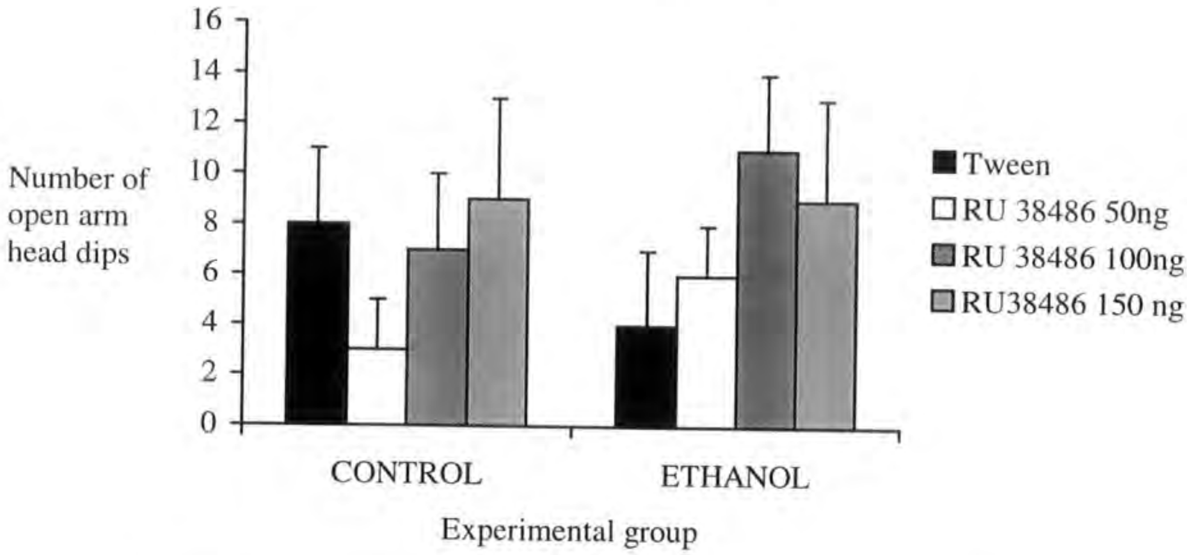


Figure 6.2 *No significant effect of diet or drug on open arm head dips*
Figure 6.3 *No significant effect of diet or drug on central square rears*

Figure 6.3

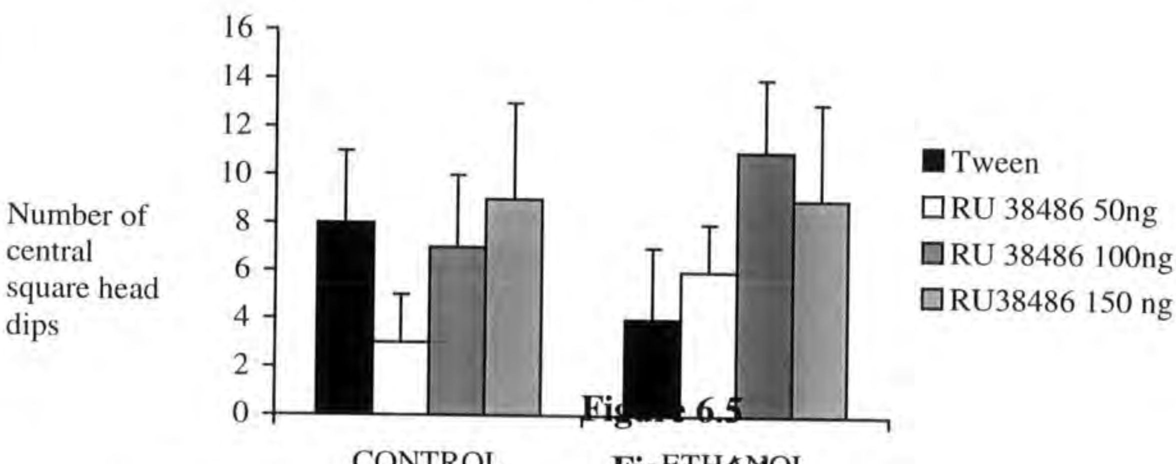


Figure 6.5

Figure 6.4

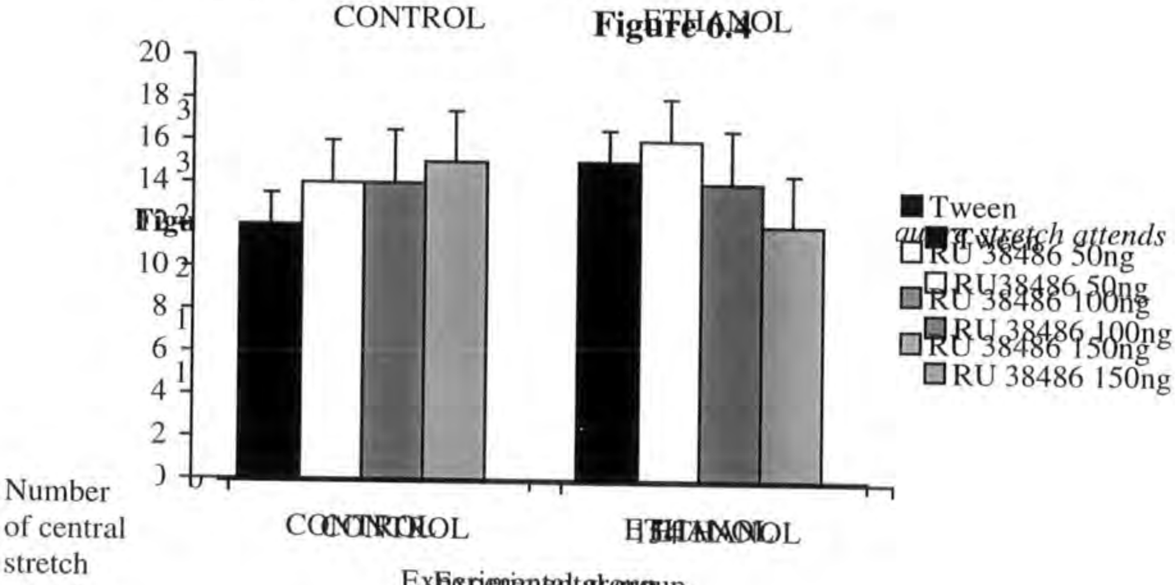


Figure 6.5 *No significant effect of drug or diet on central square head dips*

Figure 6.6

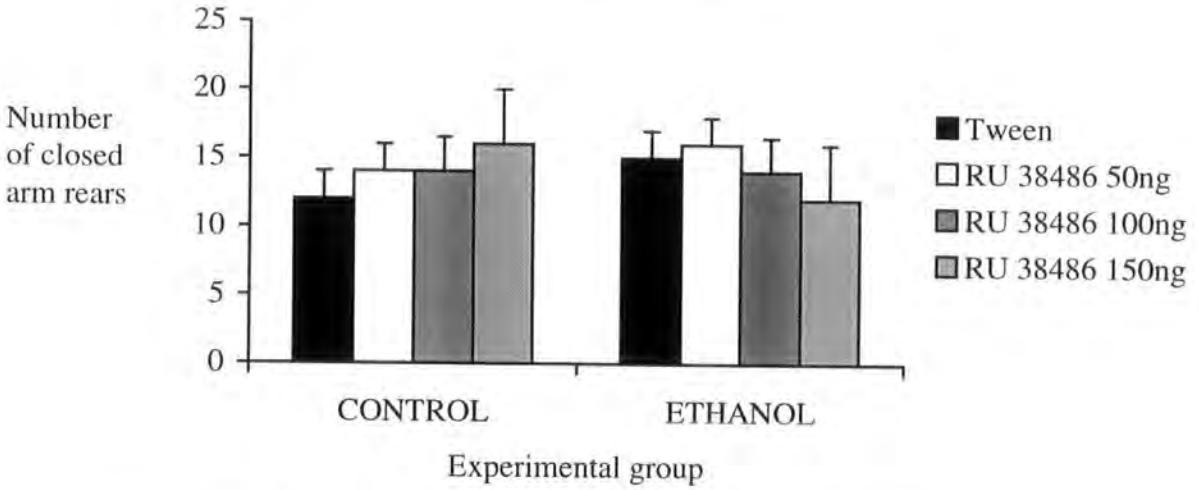


Figure 6.6 *No significant effect of drug or diet on closed arm rears*

Figure 6.7

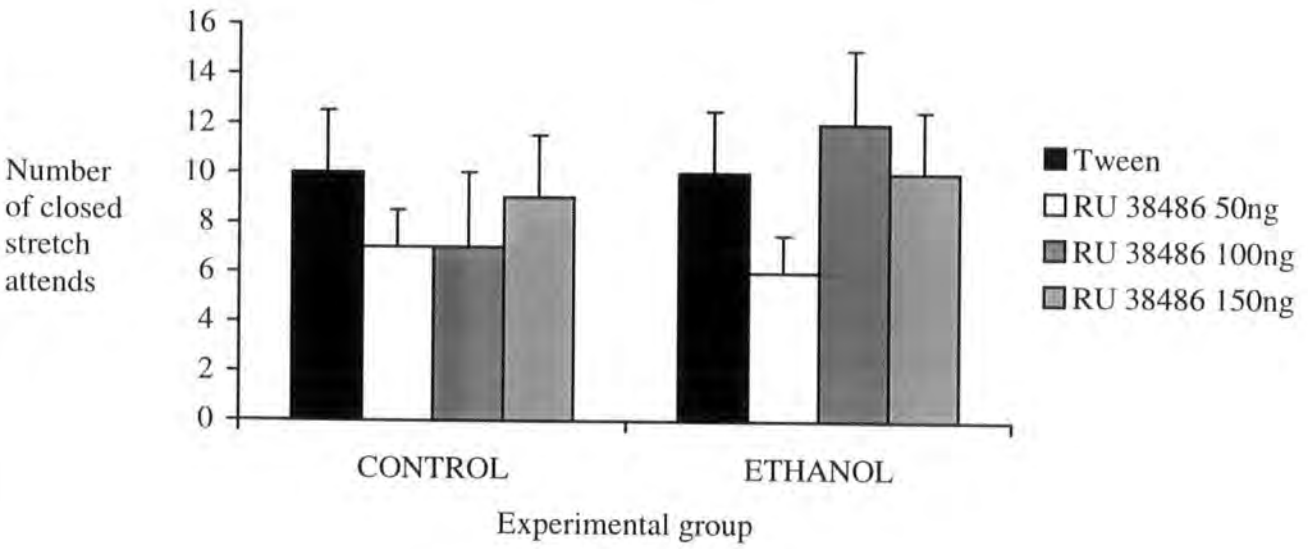


Figure 6.8

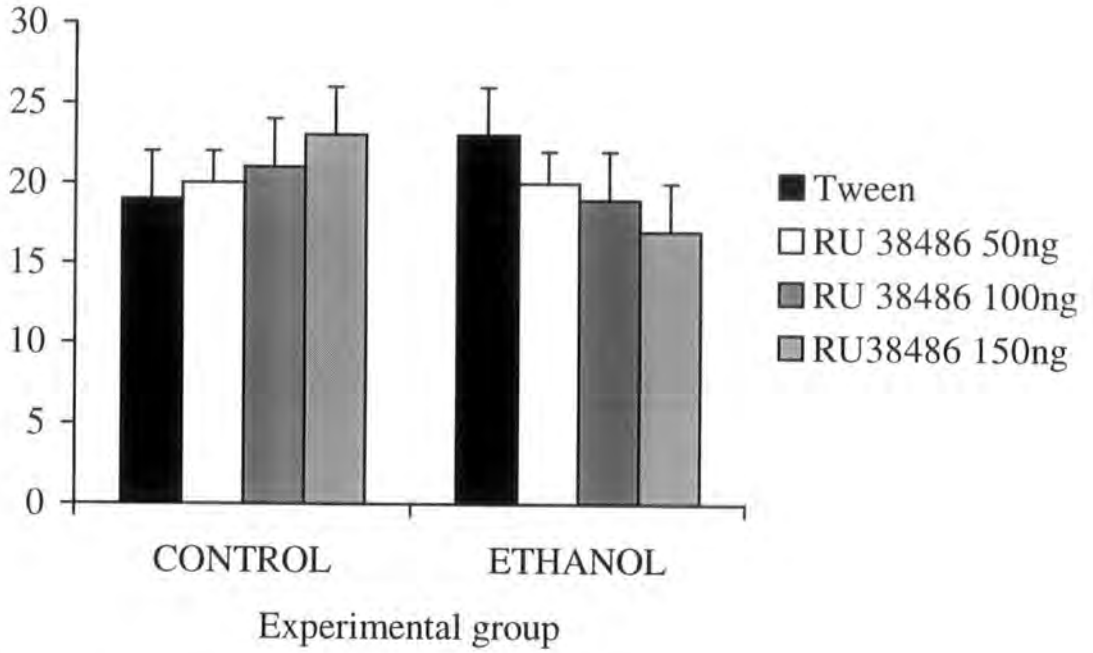


Figure 6.7 *No significant effect of drug or diet on closed stretch attends*

Figure 6.8 *No significant effect of drug or diet on closed arm entries*

Figure 6.9

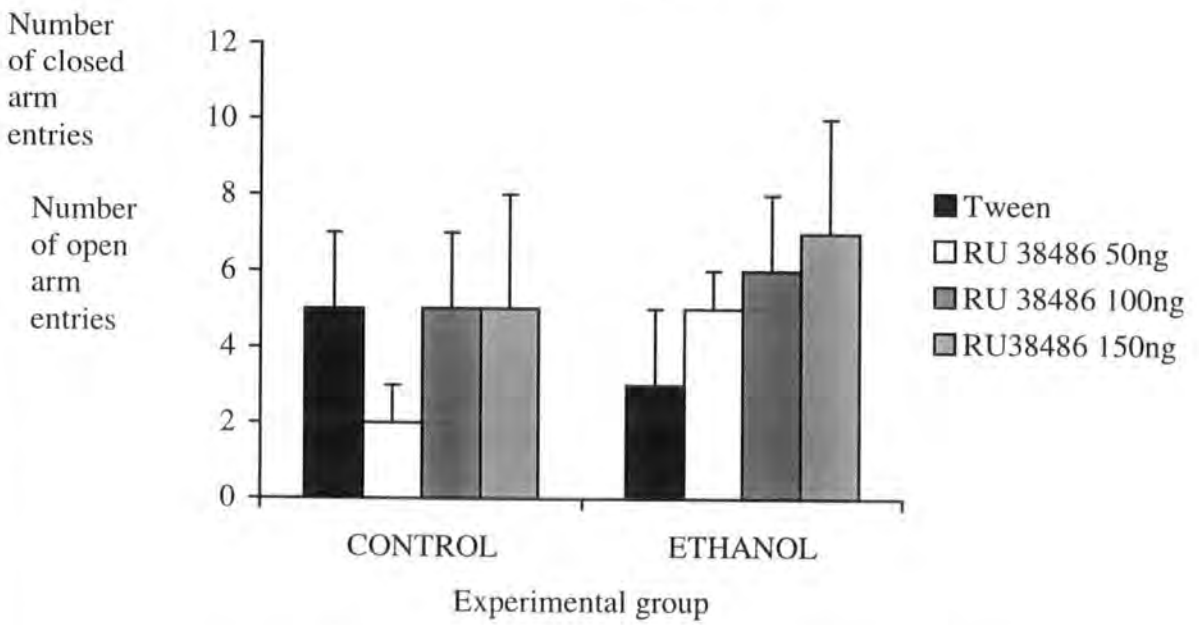


Figure 6.9 *No significant effect of drug or diet on open arm entries*

Figure 6.10

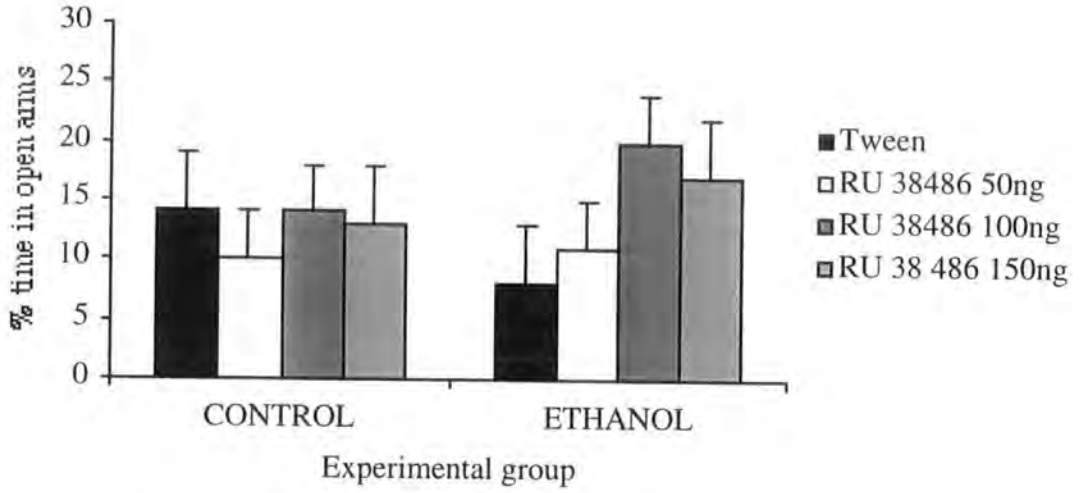


Figure 6.10 *No significant effect of drug or diet on time spent in the open arms*

Figure 6.11

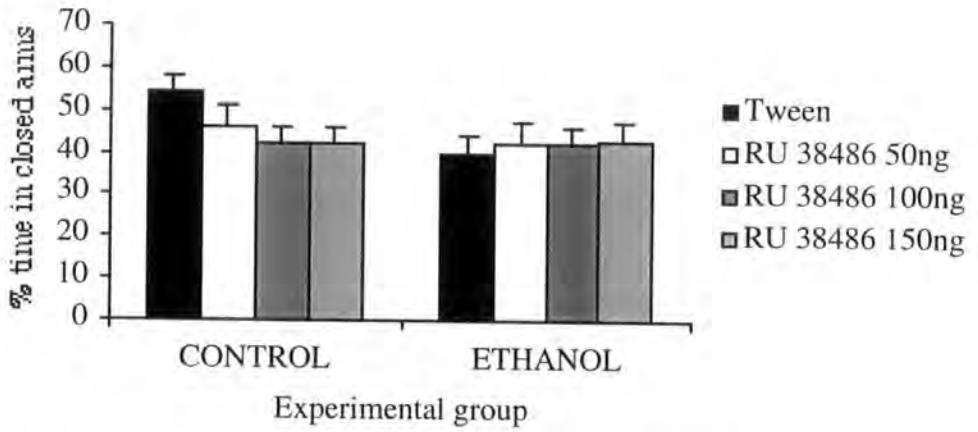


figure 6.11 *No significant effect of drug or diet on time spent in the closed arms*

Figure 6.12

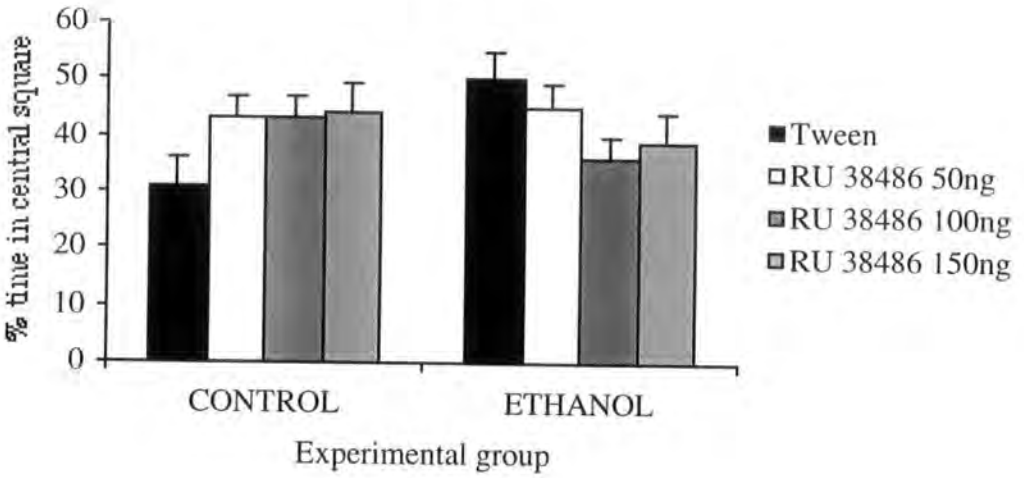


Figure 6.12 *No significant effect of drug or diet on time spent on the central square.*

Corticosterone and RU 38486 injections in normal phase TO mice

Mobile activity

Mobile locomotor activity in all groups of mice decreased with time. There was a significant effect of drug treatment ($F_{5,39} = 3$ $P<0.05$) on the total mobile activity for the whole test session. Analysis of the activity in the first five minute time bin revealed a significant increase in mobile activity ($F_{3,39}=3.7$ $P<0.05$) in the mice that had received the corticosterone injections compared with those that received the Tween vehicle ($P<0.05$), RU 28486 ($P<0.005$), and combined RU38486 and corticosterone ($P<0.05$). This data is illustrated in Figure 6.13.

The increase in activity was short lasting with the activities at 5-15 minutes showing no significant difference (Figure 6.14). In the final fifteen minutes of the session the mice treated with the combined injection showed a lower activity ($F_{3,39}=2.8$, $P<0.05$) than tween treated mice ($P<0.05$) and corticosterone treated mice ($P<0.05$) (Figure 6.15).There was no significant difference in static activity between any of the groups ($F>1$, $P>0.1$) (Figure 6.16, 6.17 & 6.18).

Rearing activity did not vary with time ($F_{5,195} = 1.5$ $P>0.05$). Corticosterone plus RU38486-treated mice showed significantly lower rearing behaviour ($F_{3,39} =3$ $P<0.05$) over the thirty minute session than those mice that received the tween vehicle injection ($P>0.05$) (Figure 6.19)

Figure 6.13

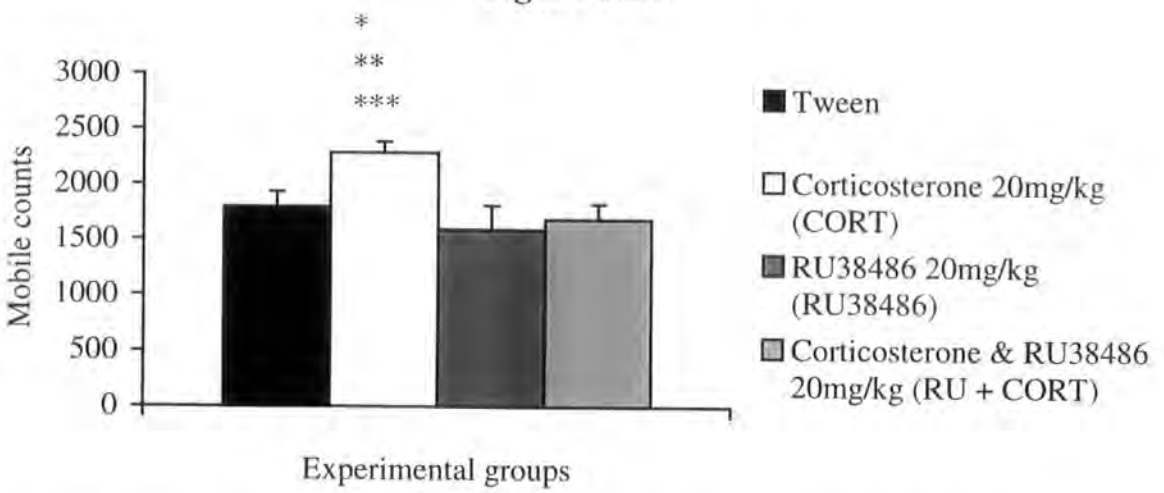


Figure 6.13 *Mobile locomotor activity counts (0-5 minutes)in naïve mice. Injections type had a significant effect ($F_{3,41}$=3.7 P<0.05). Post-hoc analysis showed that a corticosterone injection significantly increased mobile counts compared to tween vehicle (*P<0.05), RU 38486 (**P<0.005) and the combination of corticosterone and RU 38486 (***P<0.05)*

Figure 6.14

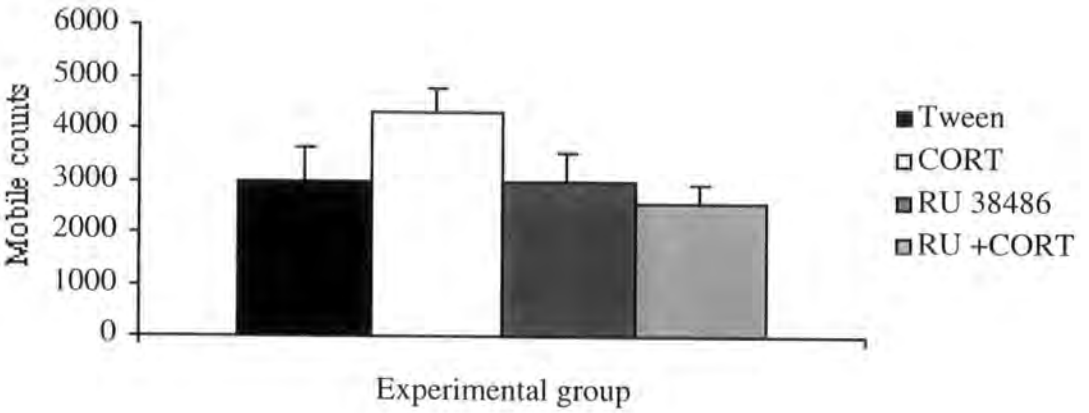


Figure 6.14 *Mobile locomotor activity counts (5-15 minutes) in naïve mice. There was no significant difference in the total mobile counts from this time bin.*

Figure 6.15

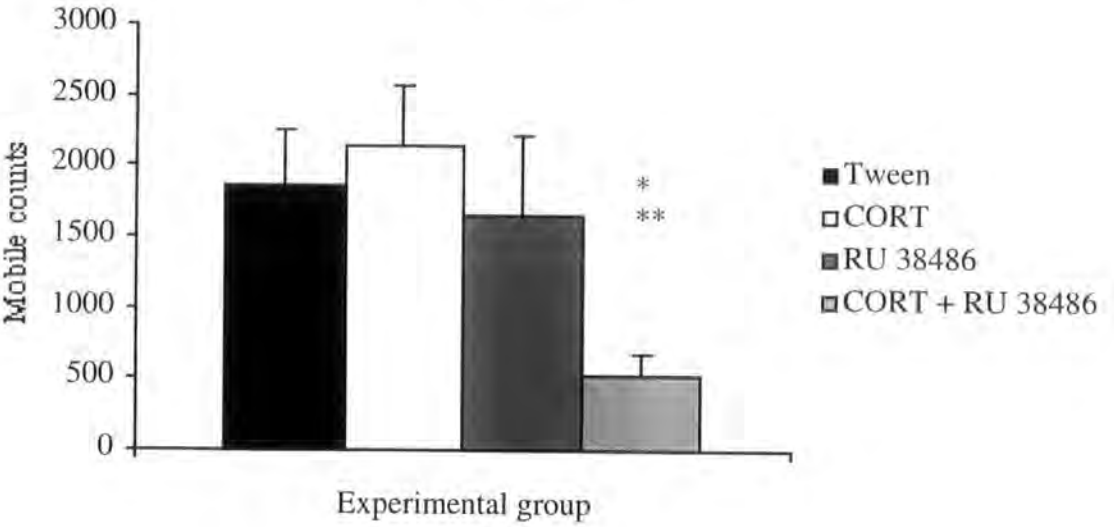


Figure 6.15 *Mobile locomotor activity counts (15-30 minutes)in naïve mice. There was a significant effect of treatment on activity (F3,39=2.8, P<0.05). Mice treated with the combination of corticosterone and RU 38486 showed significantly lower mobile counts than both tween treated mice (*P<0.05) and corticosterone treated mice (*P<0.05).*

Figure 6.16
0-5 minutes

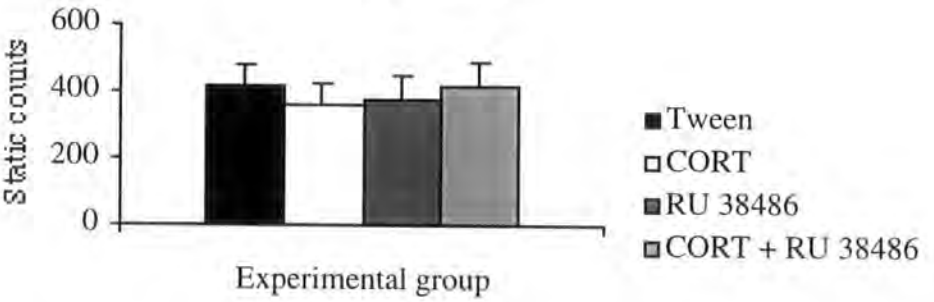


Figure 6.16 *No difference in static counts in naïve mice between the treatment groups in 0-5 minute time bin.*

Figure 6.17

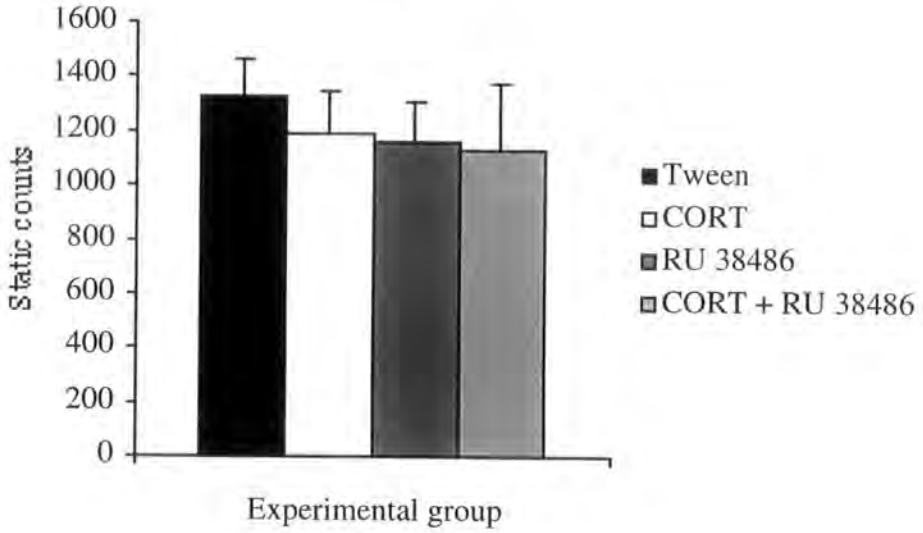


Figure 6.17 *No difference in static counts between the treatment groups in 0-15 minute time bin.*

Figure 6.18

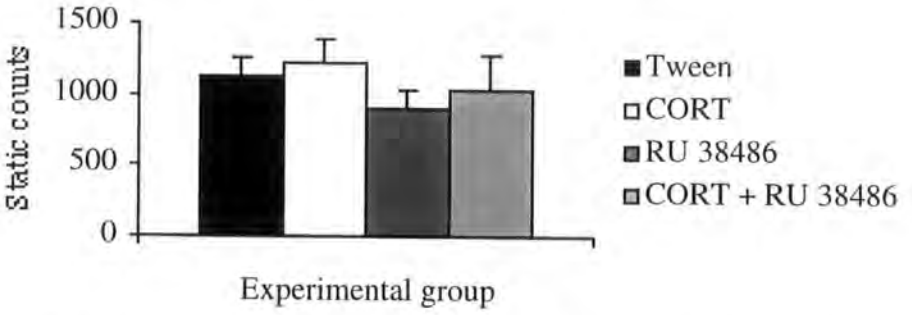


Figure 6.18 *No difference in static counts between the treatment groups in the 15-30 minute time bin (Figure 7.4c).*

Figure 6.19

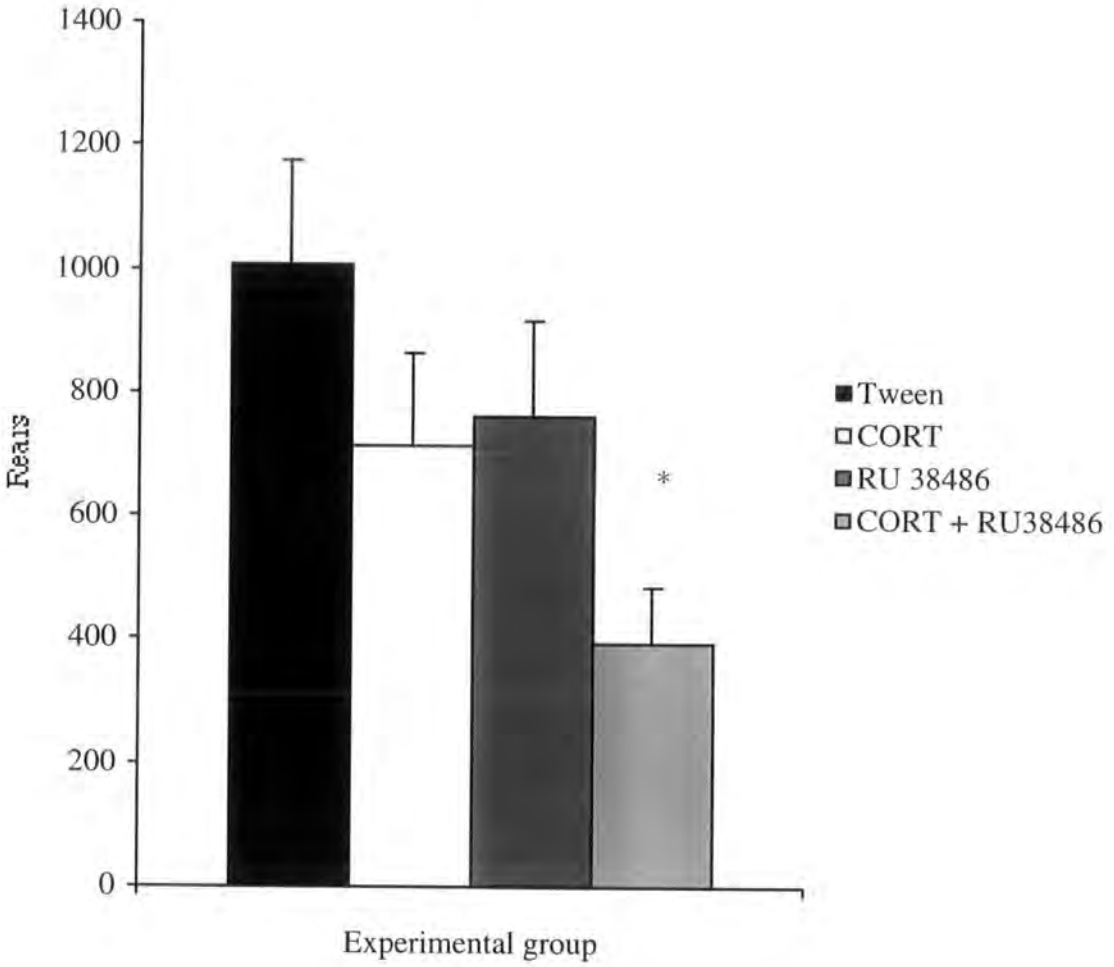


Figure 6.19 *Rearing counts for the 30 minute session. There was an significant effect of treatment on rearing counts($F_{3,39} = 3$ $P<0.05$).Post-hoc analysis demonstrated a significant difference between Tween and CORT + RU 38486 injected mice (*$P<0.05$).*

Aldosterone injections and locomotor activity

Administration of aldosterone did not effect mobile, or rearing locomotor activity. The results of mobile locomotor activity after aldosterone injections can be seen in Figure 6.20. Rearing activity is shown in Figure 6.21.

Aldosterone, at 4mg/kg, administration (Figure 6.22) altered static activity, ($F_{3,56} = 2.86$, $P<0.05$) when accessed over the entire session.

Figure 6.21

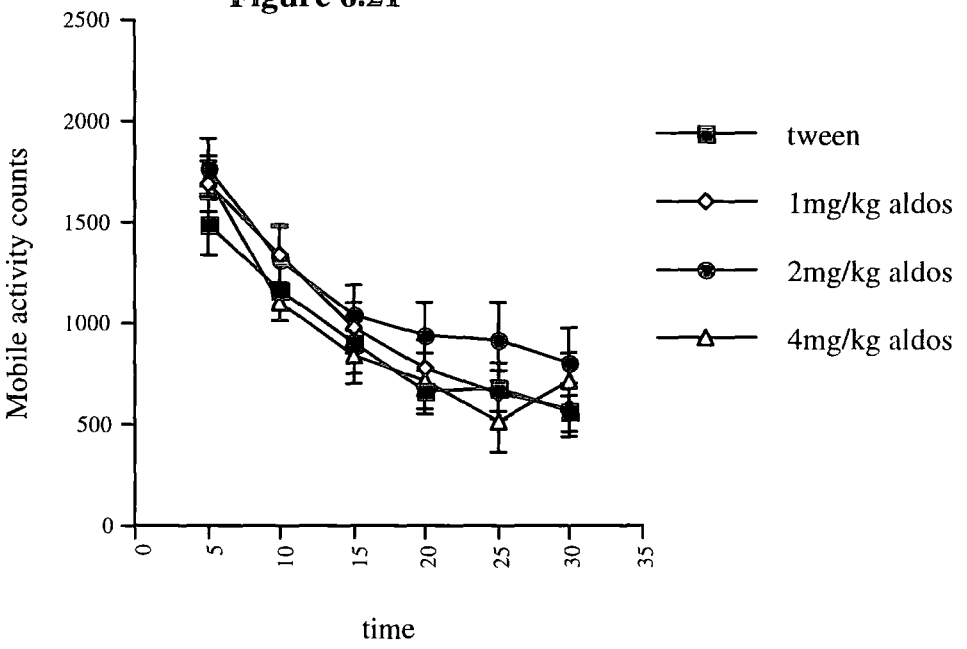


Figure 6.20.

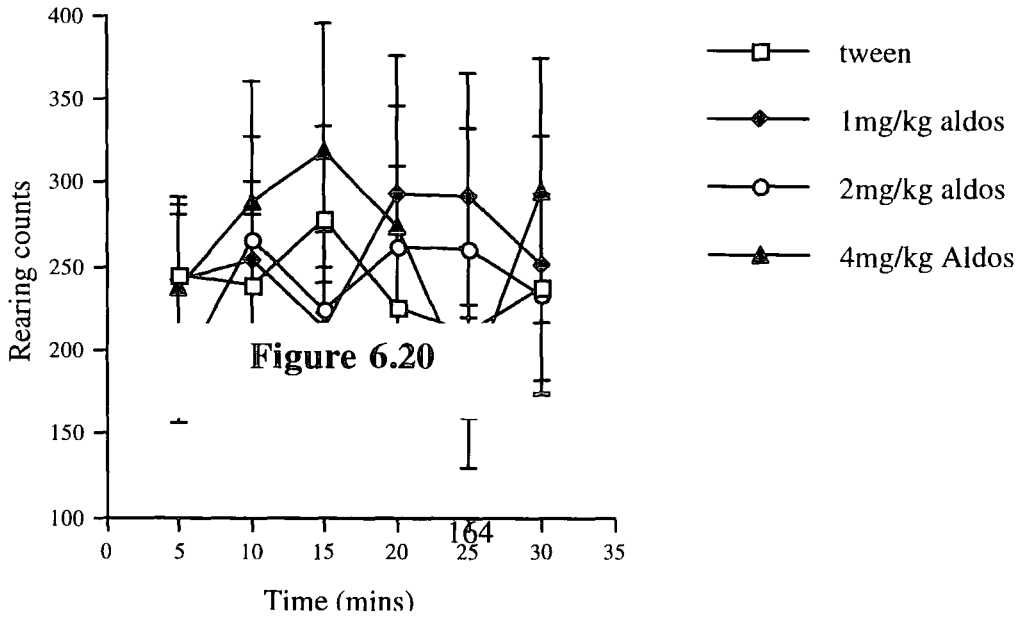


Figure 6.20

Figures 6.20 and 6.21 *No change in mobile (6.20) nor rearing (6.21) activity after administration of 1,2, 0r 4mg/kg of aldosterone (aldos). The graphs show the number of counts at individual time bins (0-5, 5-10 etc).*

Figure 6.22

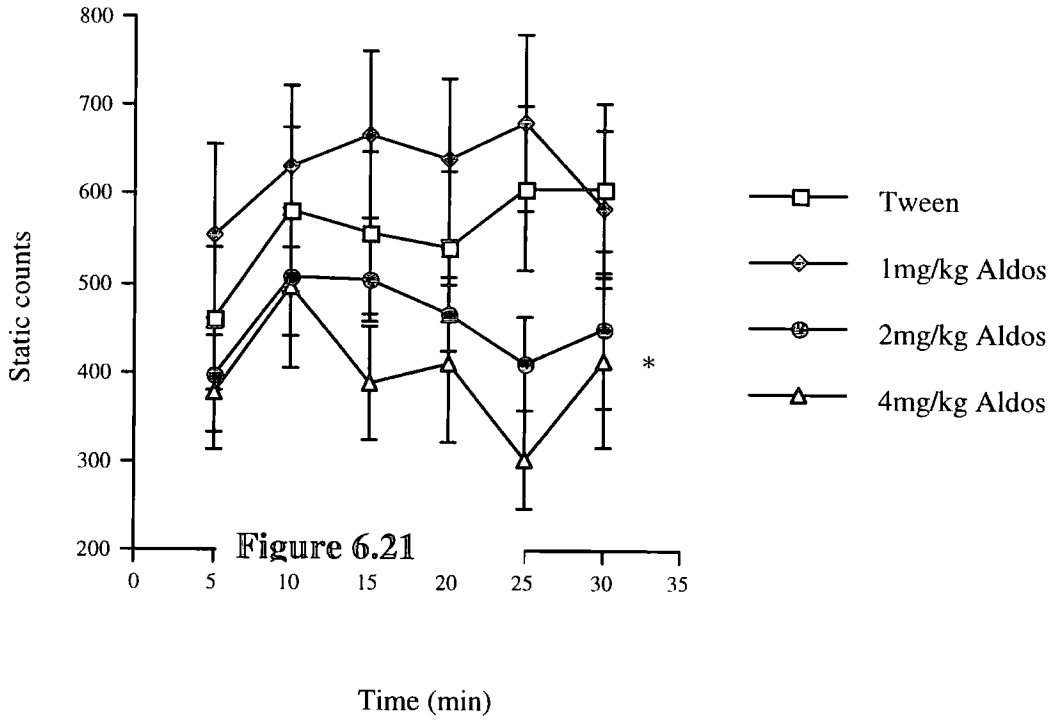


Figure 6.21

Figure 6.22 *Decreased static activity after administration of 4mg/kg of aldosterone* (*P<0.05).

Corticosterone injections after 24 hours abstinence

Static and mobile counts for all the treatment groups were analysed. Rearing behaviour was not assessed as reliable measurements of this behaviour could not be obtained in this particular experiment due to computer malfunction.

Mobile counts

The diet the mice received had a significant effect on mobile locomotor activity ($F=2$ $P<0.003$). In the control mice injection, either corticosterone or tween vehicle - but not dose, also had a significant effect ($F_{4,24} = 10$ $P<0.001$). Initially (five minute time bin) the tween vehicle injected mice showed significantly higher mobile activity than mice that received corticosterone. This effect was present at all doses ($P<0.001$, 2.5mg/kg; $P<0.001$, 5mg/kg; $P<0.001$, 10mg/kg; $P<.001$ 20mg/kg). These results are illustrated in Figure 6.23a. The injections in the alcohol treated mice did not alter mobile locomotor activity ($F=0.1$ $P=0.9$) (Figure 6.23b). The same pattern was seen over the whole thirty minute session. Injection had a significant effect ($F_{4,69} =5.5$ $P<0.0001$) with significant differences between tween-injected and each of the individual corticosterone doses (all at $P<0.001$) for the control diet mice. Whereas in the ethanol treated mice there was no difference between the different injection groups (Table 6.1).

Figure 6.23a

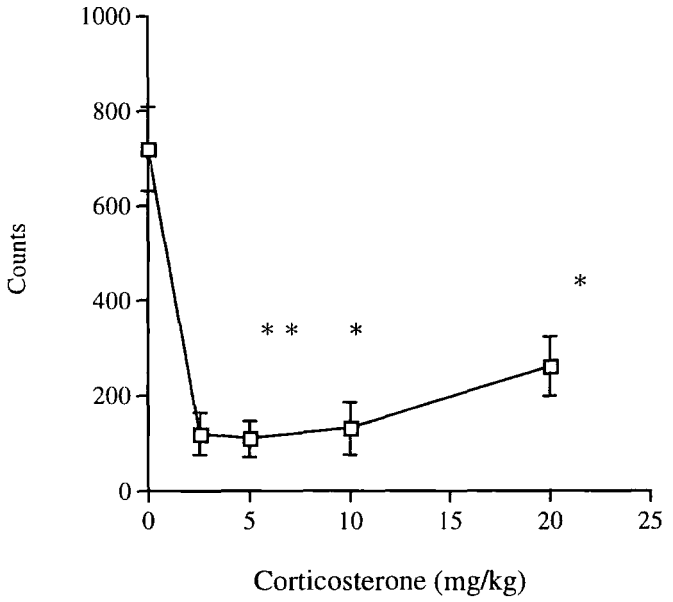


Figure 6.23b

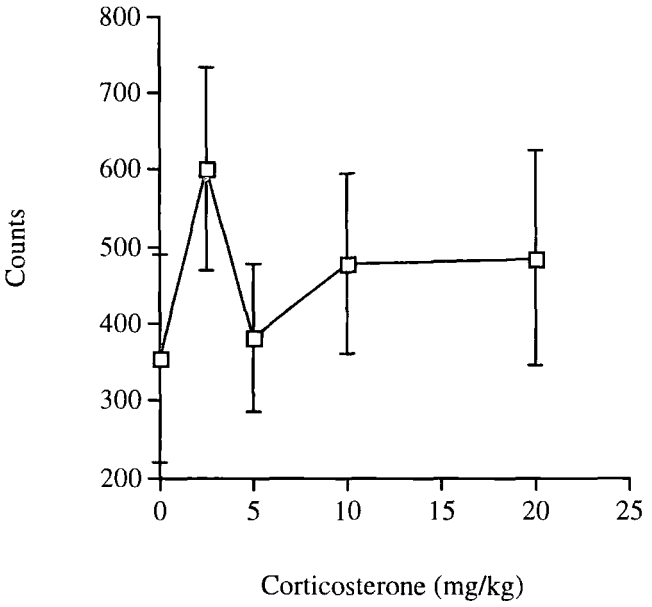


Figure 6.23 (a) *Mobile activity for the(first five minute time bin) in control diet mice. Corticosterone injections significantly reduced activity compared to vehicle injections (*P<0.001)* **(b)** *Mobile activity counts (first five minute time bin) in alcohol diet treated mice that received corticosterone injections. There was no difference in activity in the different drug doses.*

Table 6.1

Treatment	Tween	2.5mg/kg	5mg/kg	10mg/kg	20mg/kg
Control diet	2472±280*	601±187*	596±174*	506±120*	857±259*
Ethanol diet	1363±522**	964±173	1239±306	852±160	1046±326

*Mobile activity for the whole 30 minute session was decreased by administration of corticosterone compared to vehicle injections in control mice (*P<0.001).Vehicle-injected alcohol-treated mice showed lower locomotor activity compared with vehicle-injected control mice (**P<0.05).*

Static counts

Static counts for the whole session were higher in the alcohol than the control group ($F_{1,72} = 8$ $P<0.005$). Treatment type was also significant ($F_{9,64} = 3$ $P<0.005$). There was a significant interaction between diet and treatment ($F_{4,64} = 2.5$ $P<0.05$). There was no significant difference between control and ethanol diet treated mice that received the vehicle injections. Control and alcohol mice that received the lowest (2.5mg/kg) dose of corticosterone did not show significantly different static counts. Alcohol treated mice showed higher static activity than control diet mice when given corticosterone doses of 5mg/kg ($P<0.01$), 10mg/kg ($P<0.01$) and 20mg/kg ($P<0.02$). These results are illustrated in Figure 6.24

Figure 6.24

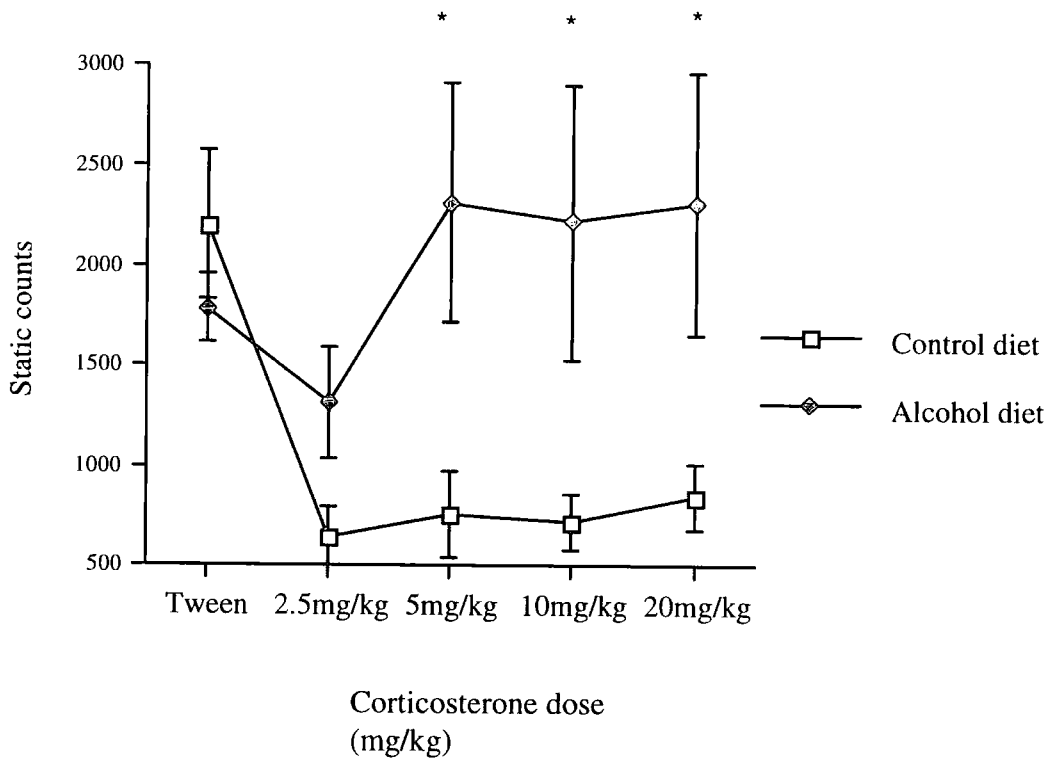


Figure 6.24 *Graph of static activity counts in a 30 minute test session after corticosterone injections and liquid diet treatments. No significant difference in the static counts of mice that received tween injections or 2.5 mg/kg corticosterone injections. 5 mg/kg corticosterone significantly increased static counts in alcohol diet treated mice(*P<0.001). 10 mg/kg corticosterone significantly increased static counts in alcohol diet treated mice(*P<0.001). 20 mg/kg corticosterone significantly increased static counts in alcohol diet treated mice(*P<0.001).*

Corticosterone injections after 6 days abstinence

Diet treatment had a significant effect on mobile activity ($F_{9,65} = 7.6$ $P<0.001$). Control mice that received tween injections had higher mobile activity than alcohol treated mice given tween injections ($P<0.01$). This difference in activity was present at the start of the session, the first five minute time bin (Figure 6.25) and continued over the entire session (Figure 6.26). Although the tween injections had an effect on mobile activity of the control mice compared with alcohol-treated mice, none of the corticosterone doses altered activity when comparing between diet treatments or within diet treatments. The absence of effect was seen initially (first five minute time bin) and over the whole 30 minute session. The mobile activity counts for 10 mg/kg are not displayed owing to computer error during data acquisition.

Figure 6.25

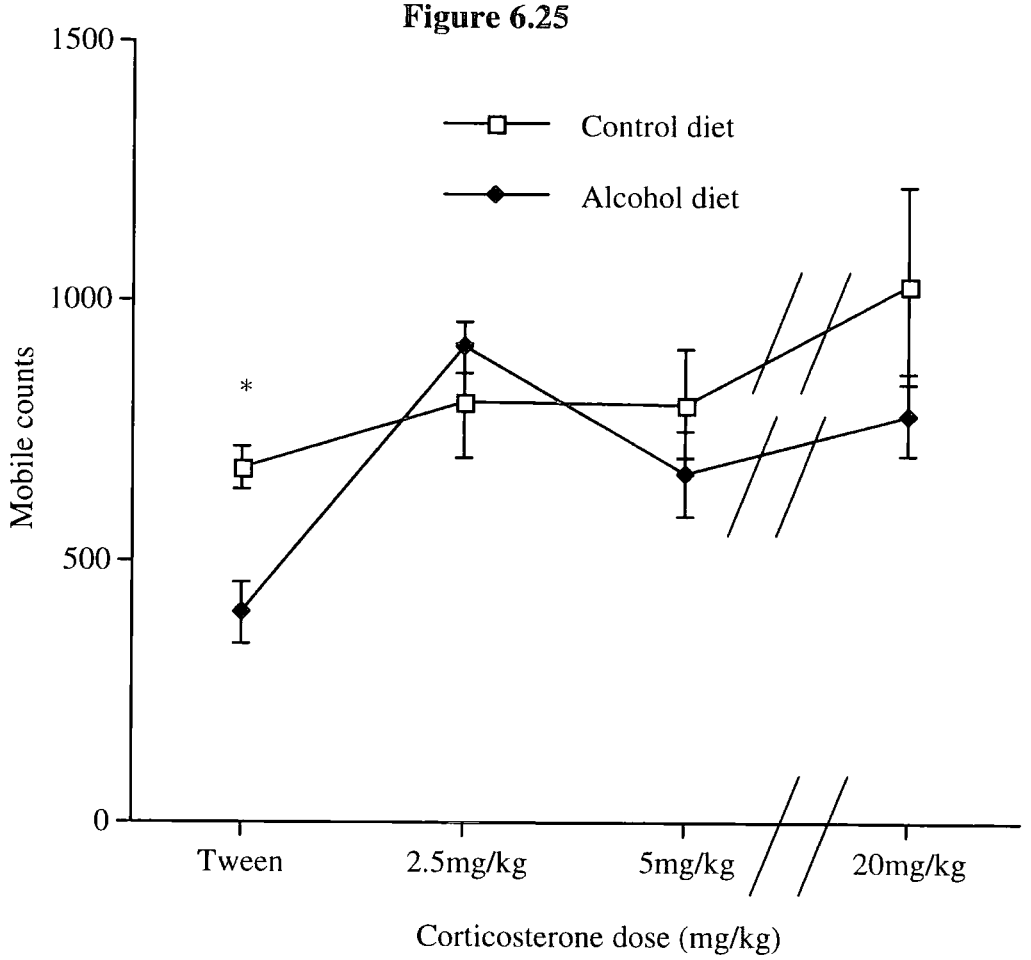


Figure 6.25 *Mobile locomotor activity during the first five minutes of activity testing. Control mice that received tween vehicle injections showed an increased activity compared with alcohol diet treated mice that received tween vehicle injections (*P<0.01).*

Figure 6.26

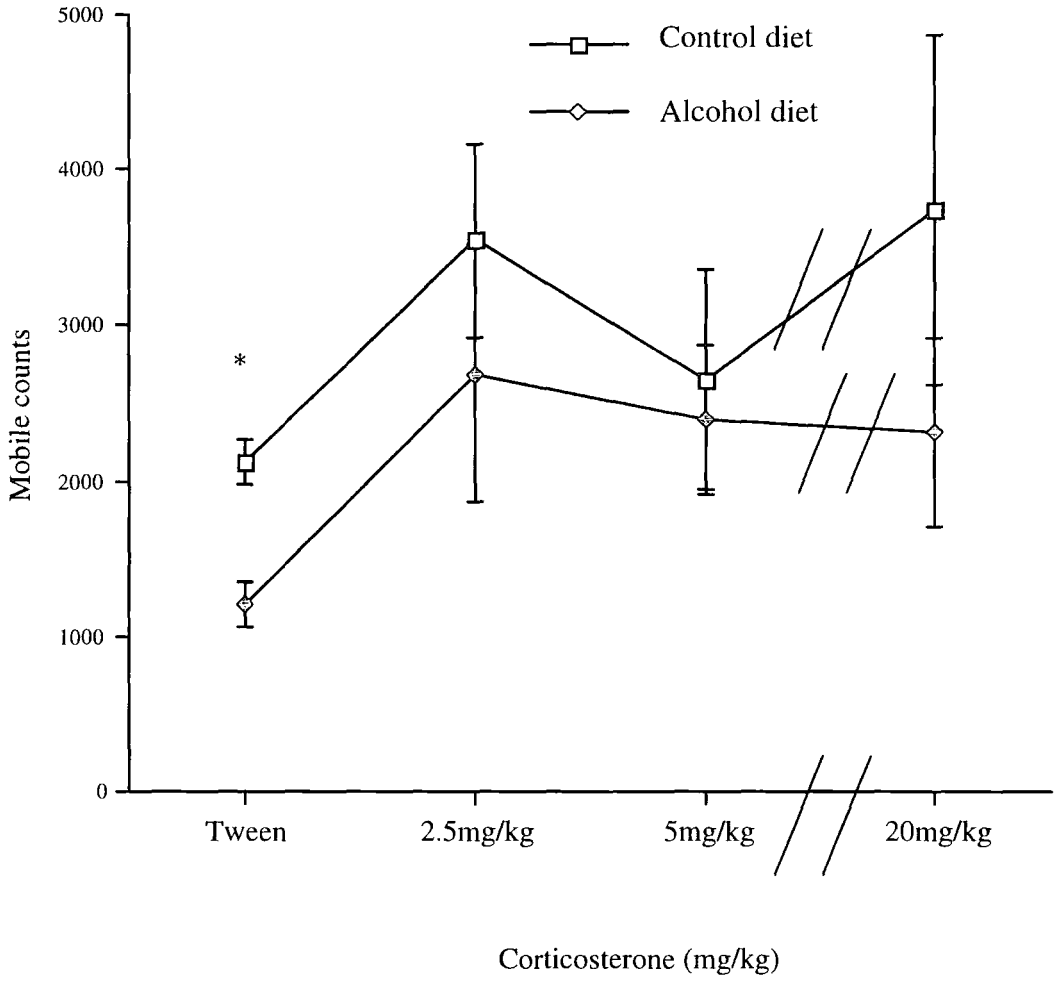


Figure 6.26 Mobile locomotor activity during the entire testing session. Control mice that received tween vehicle injections showed an increased activity compared with alcohol diet treated mice that received tween vehicle injections (*P<0.01).

Comparison of 24 hour and 6 day withdrawal mobile activity.

Figures 6.27 shows a comparison between the mobile locomotor activity of the control mice at 24 hours withdrawn and 6 days withdrawn (Figure 2.27a) and the mobile locomotor activity of the alcohol treated mice at 24 hours withdrawn and 6 days withdrawn (Figure 2.27b). The mobile activity of the control mice receiving corticosterone injections showed lower mobile activity at 24 hours withdrawn compared to their 6 day alcohol abstinence counterparts ($P<0.001$). There was no difference to mobile locomotor activity in the alcohol diet treated mice at the different time points.

Figure 6.27a

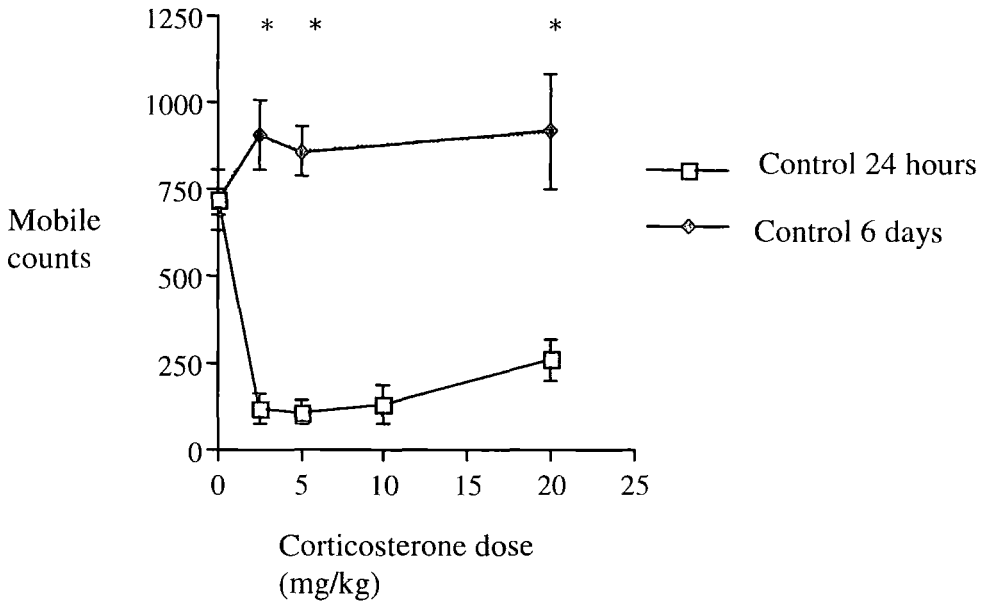


Figure 6.27b

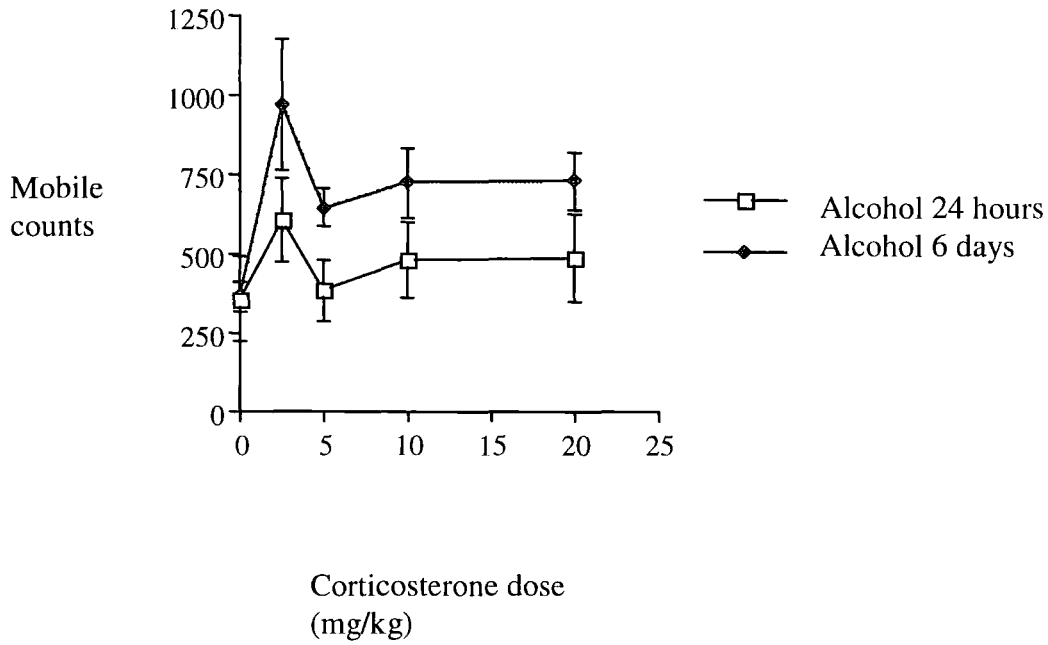


Figure 6.27 *Comparison between mobile activity in the first five minutes in (**a**) control diet treated mice (6 days and 24 hour withdrawal) and (**b**) alcohol diet treated mice (6 days and 24 hour withdrawal).24 hour control diet treated mice had significantly lower activity than 6 day withdrawal control mice for all corticosterone doses (*P<0.001).*

Corticosterone levels

Following the 23 day liquid diet treatment and six days of withdrawal the levels of total blood corticosterone levels in control and alcohol diet treated mice did not differ (Figure 6.28). The levels of free corticosterone (Fig 6.29) were significantly higher in alcohol treated mice compared to control mice ($P<0.05$).

There was no difference in corticosterone levels in the cortex (Fig 6.30) or in the striatum between the two treatment groups (Fig 6.31). In the alcohol-treated mice hippocampal corticosterone levels were significantly increased compared with control treated mice($P<0.001$) (Figure 6.32).

Figure 6.28

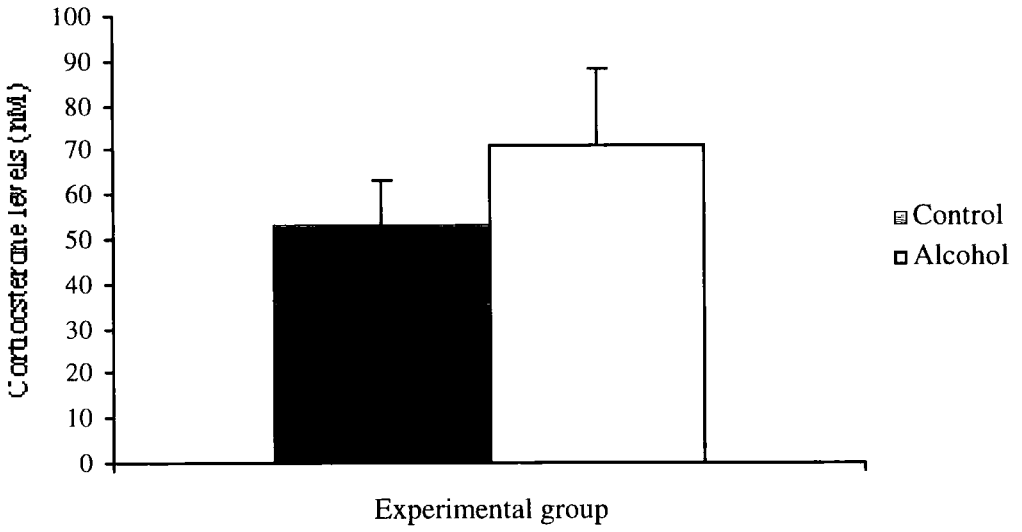


Figure 6.28 *Total blood corticosterone levels in control and alcohol-treated mice. There was no significant difference between the two treatment groups.*

Figure 6.29

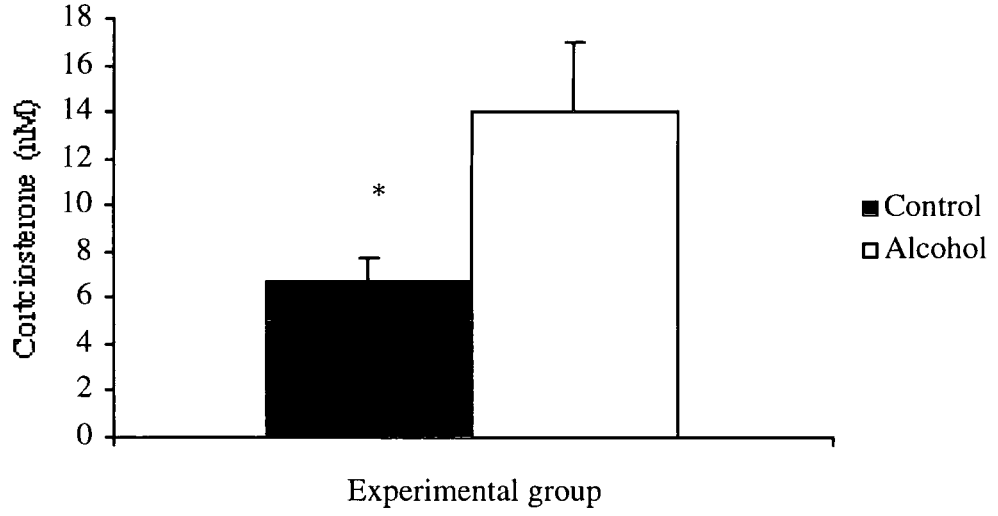


Figure 6.29 *Free blood corticosterone levels in control and alcohol-treated mice. The free corticosterone levels in alcohol diet treated mice were significantly higher than control diet treated mice (*P<0.05)*

Figure 6.30

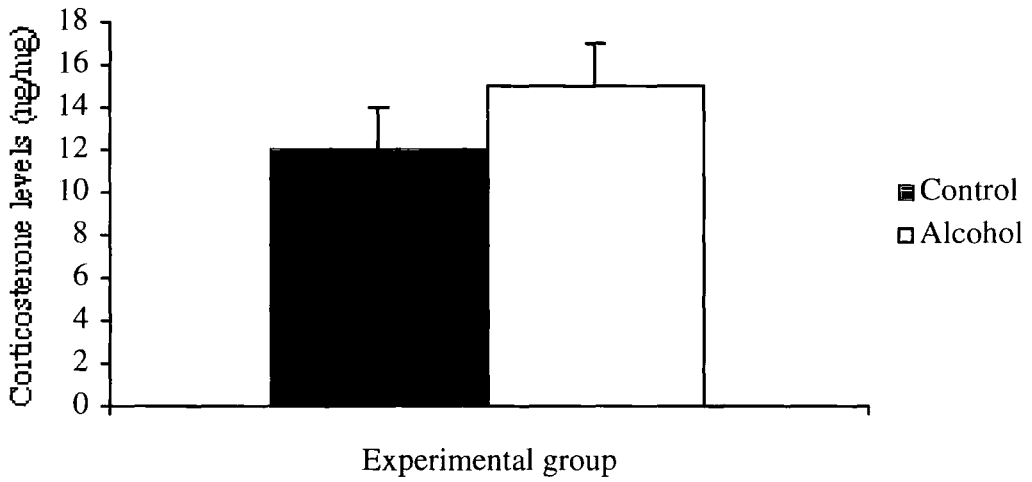


Figure 6.30 *Corticosterone levels in the cortices of alcohol-treated and control mice. There was no significant difference between the two treatment groups.*

Figure 6.31

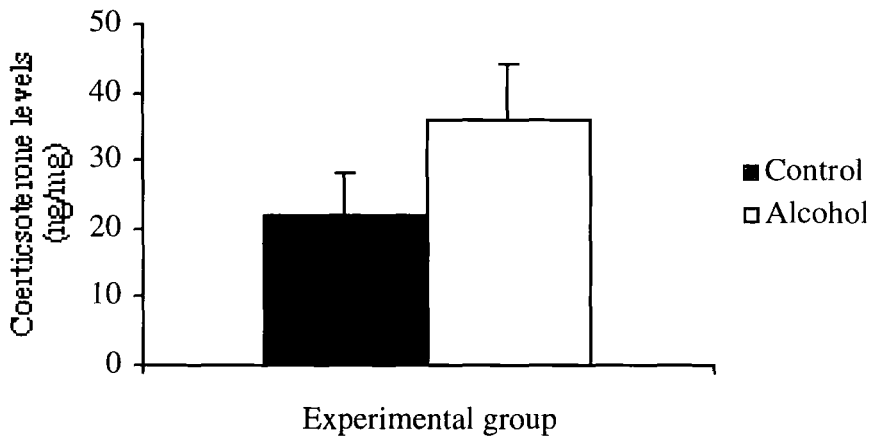


Figure 6.31 *Corticosterone levels in the striatum of alcohol-treated and control mice. There was no significant difference in the levels measured in the two treatment groups.*

Figure 6.32

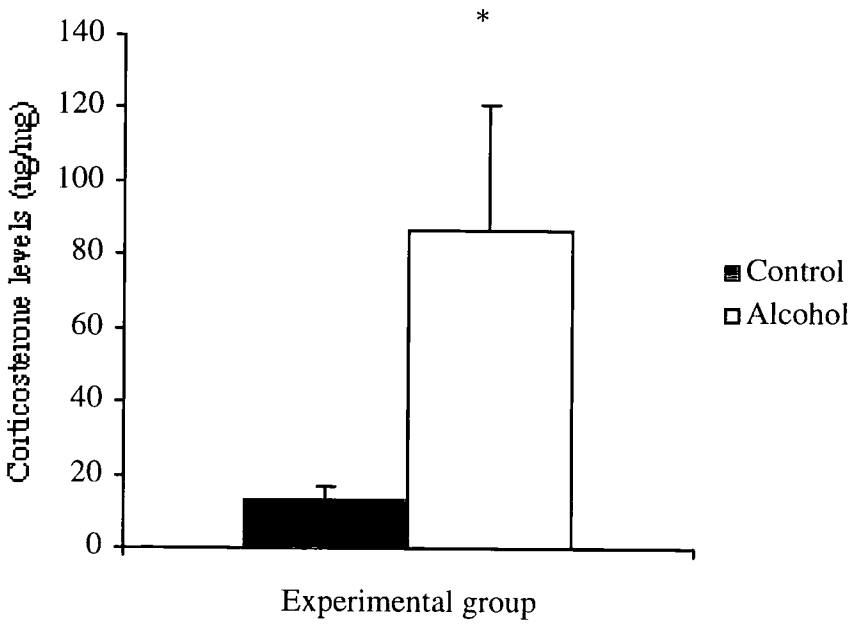


Figure 6.32 *The levels of corticosterone in the hippocampus after control or alcohol liquid diet. Mice that received alcohol liquid diet had significantly higher hippocampal corticosterone levels than mice that received control liquid diet (*P<0.002).*

Discussion.

Locomotor activity

The administration of corticosterone to untreated mice produced a transient increase in mobile locomotor activity, apparent during the first five minutes of measurement. This increase in mobile activity ceased after five minutes. Previously reported corticosterone-induced increases in activity, that were measured in rats, started between five and ten minutes after injection and disappeared within 60 minutes of drug administration (Sandi et al., 1996). It is possible that corticosterone-induced activity in mice follows a more rapid time course than it does in rats.

The mice that were treated with RU38486 and corticosterone in combination reduced lower mobile activity and reduced rearing behaviour in the last half of the test session (15-30 minutes). The RU38486 itself had no effect. This observation, though not conclusive, would appear to suggest that corticosterone acting at mineralocorticoid (MR) receptors has decreased locomotor activity. RU38486 blocks glucocorticoid receptors. When administered in combination (RU38486 and corticosterone) the receptors available to the administered corticosterone should only be the MR. However, this is not supported by the results of the locomotor activity experiments conducted after the administration of the MR agonist aldosterone, none of the aldosterone doses altered locomotor activity. However, aldosterone does not interact with all the central MRs. Although MR can be defined by a high affinity for aldosterone, they also have a high affinity for corticosterone. It has been proposed that the two ligands do not compete for the same central receptors. Ligand transporters, and enzymatic selection have been proposed to confer

ligand receptor specificity in different brain regions (Sekle, 1997). With hindsight, aldosterone was not an ideal ligand to assess the influence of MR receptors on locomotor activity. A better choice could have been to study the effects of a selective MR antagonist, such as spironolactone on locomotor activity.

When the effects of corticosterone on the alcohol-treated mice were examined appeared to contradict the initial experiments. There was no corticosterone-induced increase in activity in the mice that were tested 24 hours after the removal of the liquid diet in either the control fed mice or the alcohol diet fed mice. The control mice showed a reduction in locomotor activity for all the doses of corticosterone and this effect was seen for the entire testing period. This change could in part be explained by the time at which the animals were tested. The untreated mice were tested under their light phase and the treated mice were tested under their dark phase. The alcohol-treated mice were tested under dark phase because this is the period of time at which mice are active and so was believed to be the best period for behavioural testing. In addition to this difference the untreated mice were injected and immediately had their activity monitored whereas the liquid diet treated mice had a ten minute habituation to the chamber before injection and the measurement of activity. The habituation time was included as prior to the experiment it was assumed from the previous results that there would be an increase in activity and so by giving the mice a short time to habituate a lower baseline of activity would be achieved, so any increase in activity would be easier to detect. The lack of change in activity of the alcohol-treated mice can therefore be explained by those two factors, measurement at a different time of day and the inclusion of a period of

habituation at the start of the experiment. The effect of corticosterone on the control animals after 24 hours withdrawal (a reduction in activity) cannot be so easily explained.

The reduction in activity seen was likely to be an artefact of the liquid diet treatment. When the mice undergo the liquid diet treatment the control mice only receive the same volume of diet as the alcohol treated mice will consume. The mice on the alcohol diet also tend to consume the diet steadily over the 24 hour period, but the mice on the control diet consume their diet upon presentation. The limited access to the diet causes excessive consumption of the standard laboratory chow when the treatment schedule has finished. Firing rates, in alcohol-treated mice, in the VTA are decreased at twenty-four hours withdrawal (Bailey et al., 1997). This altered neuronal state could explain the difference in responses to corticosterone injections after binge eating in the control and alcohol-treated groups.

In alcohol treated mice there was an increase in static counts at corticosterone doses of 5 mg/kg and above. This could have been an increase in either grooming, digging sawdust or an artefact of the reduced activity in the control mice. The decrease of mobile activity in the control mice makes the most plausible explanation in that the lower static counts seen are a result of sedation rather than an increase in activity in alcohol-treated mice.

The results of the activity measurement after six days abstinence showed increased mobile activity in control mice after they received injections. The difference in mobile activity between the two groups following vehicle injection has not been shown before. The work by Manley & Little (1997) on the response of chronic liquid diet treated mice (using the same diet treatment schedule) to

amphetamine and cocaine administration showed no effect of vehicle injections on locomotor activity when the activity was measured at the same time point. Again this difference in results may be due to the testing being done in different photo-periods. The experiments performed by Manley were completed during the light phase unlike these experiments that were performed during the dark phase.

The plus maze

No increases in anxiety-like behaviour were seen in ethanol treated mice when compared to control treated animals whereas, a reduction in, entries onto, and time spent on, the open arms of the maze, has been demonstrated during acute withdrawal (16hrs) in mice. During alcohol acute withdrawal increases in plasma corticosterone have been demonstrated (Tabakoff et al., 1978) these are reported to disappear after 24hrs.

Long lasting changes in the HPA axis have been demonstrated (e.g. Post, 1992; Buwalda et al., 1999) after stress and after the inducement of consistent or repeatedly high levels of corticosterone. The changes have been shown following two short-lasting episodes of intensive stress in the rat model of social defeat (Buwalda et al, 1999). One week after the defeat Buwalda and colleagues showed a decrease in hippocampal and hypothalamic GR binding but no change in MR binding. After three weeks MR binding was increased in the hippocampus whereas GR binding had returned to control levels.

The alterations in corticosterone levels in withdrawal could be expected on the previous evidence to produce a long lasting change in HPA function and alterations in corticosterone receptor function. Whether a change in HPA function could be demonstrated by the use of the elevated plus maze and the GR antagonist RU38486 is not clear. No significant change between any of the RU

38486 groups was detected. The previous plus maze studies that demonstrated an anxiolytic action of RU38486 used a pretest stress and only after the pretest stressor was the drug effective as an anxiolytic. In the present study the administration of alcohol was designated to be the pre-test stressor, albeit an ineffective one. Glucocorticoids have been reported to have opposing effects on anxiety-related behaviour. Adrenalectomy produces anxiogenic effects, whereas corticosterone administration produces anxiolytic effects (File & Pellow, 1985). However fear-motivated immobility has been reported to be abolished by adrenalectomy and restored after corticosterone replacement (Veldhuis et al., 1982). Korte and colleagues (1996) have suggested a biphasic effect of corticosterone mediated by different corticosterone receptor subtypes at different locations. Fahkle and Eriksson (2000) have also recently shown that corticosterone implants in only selected brain regions (the ventral striatum) could alter alcohol intake. The present study was limited by the use of only the glucocorticoid antagonist. It would have been preferable to investigate the effects of a mineralocorticoid antagonists as well. An avenue for investigation of corticoid receptor function - radioligand and autoradiography was considered to further examine potential alterations during absence.

The evidence from this study does not provide much information on the state of glucocorticoid receptors after alcohol treatment. The study does show that after six day withdrawal following chronic alcohol treatment there is no difference in anxiety related behavioural on the elevated plus maze. The simplest and most direct measure of corticoid receptor state (affinity constant; Kd and binding density; Bmax) would be to measure the receptors directly using homogenate radioligand binding. This is not as simple as it would appear for a

number of reasons. In vitro binding studies are commonly used to quantify soluble GR levels and levels of MR.

A problem encountered when attempting to measure these receptors is the difficulty in removing the endogenous ligand. The normal procedure for removal of endogenous ligand in a binding assay for membrane bound receptors is to wash the ligand away using multiple homogenization and centrifugation steps. It is not possible to do this when measuring corticosterone receptors as they are soluble. Removal of endogenous corticosterone is normally achieved by Adrenalectomising the animal, this procedure will remove circulating corticosterone. The assay is performed 24 hours after the adrenalectomy, i.e. when there is no endogenous ligand remaining (Spencer et al., 1990). However, the measurements of corticosterone binding made after this may not present a true picture of the state of the receptors. Adrenalectomy-induced increases in GR binding sites occur in two distinct phases (McEwen et al., 1974). The first phase occurs between 2 and 16 hours after surgery and is thought to mirror the clearance of endogenous steroids. A second phase is 18-24 hours after surgery and is believed to be the result of de novo synthesis of receptors. These times may vary between laboratories owing to slightly different techniques or different rodent strains (O'Donnell et al., 1995). This means that when measuring receptor levels after adrenalectomy, there is only a two hour window to measure the receptors, i.e. when the endogenous ligand has been cleared but before the new receptors are made. It seems unlikely that an organism's response to adrenalectomy will in reality provide such a window. This would mean that any binding assay performed on adrenalectomised animals would have to be qualified. The effects of alcohol on corticosterone receptors during and after

alcohol administration is an important question, the effects of alcohol and adrenalectomy would provide very limited information. For these reasons biochemical measurements of corticosterone receptors were not attempted.

Corticosterone levels

Increased levels of free, but not total, circulating corticosterone were found in the mice that were withdrawn for six days from alcohol liquid diet. The levels of corticosterone found in the hippocampus of alcohol-treated mice were elevated. Long-term alcohol intake has been reported to produce morphological and neurochemical abnormalities in the CNS of both humans and experimental animals (Charness, 1983). Neuronal loss of between 10 and 30% in CA1, CA3 and the granular layer of the dentate gyrus has been reported after long-term alcohol intake (Cadete et al., 1988). These changes have been found in animals withdrawn from alcohol and in some cases the deterioration has in fact increased during the abstinence phase (Cadete et al., 1991; King et al., 1988). Long-term alcohol consumption (13 months) followed by a six week withdrawal period showed that alcohol-treated rats were cognitively impaired relative to both animals on continuous alcohol consumption and age matched controls (Lukoyanov et al., 1999). It is possible that the increased hippocampal corticosterone levels reported in this study could provide a mechanism by which these cognitive impairments and neurodegeneration develop. Stress and increased glucocorticoid levels are now believed to have specific effects on cognitive function. Corticosterone has been shown to produce deficits in memory in animal models (Lupien & McEwen., 1997). Stress induced hippocampal atrophy is believed to be caused by excitatory amino acids acting via NMDA receptors in concert with glucocorticoids (McEwen, 1998).

In addition to the important role the hippocampus has in learning and memory, the hippocampus is also involved in the regulation of HPA activity (Jacobson & Sapolsky, 1991). Hippocampal influence on the HPA is largely inhibitory, acting to shut down HPA activity rather than being a site for the control of negative feedback. Thus impaired hippocampal function could lead to increased levels of HPA axis activity; increased release of CRF, ACTH and corticosterone.

A degree of control of alcohol intake by the HPA has been shown in this thesis and by others (see Chapters One to Five), it is therefore not unreasonable to link impaired HPA regulation with alcohol intake. This increase in hippocampal corticosterone was observed in abstinent mice, which raises the question could this regionally specific rise in corticosterone be part of the mechanism governing the propensity of alcoholics to relapse? It has been suggested that a protracted withdrawal syndrome that may have detrimental long-term effects on the psychological functioning and rates of relapse in alcohol dependent patients in treatment (Carlsson et al., 1996). De Soto et al., (1985) studied the symptomatology in alcoholics in various stages of abstinence, showing that in some patients affective and vegetative symptoms remain present for long periods of time. The elevated corticosterone levels reported in this chapter may help explain the extended withdrawal syndrome that has been reported.

Recently, long-term changes in the HPA axis after chronic alcohol administration have been reported by other researchers (Rasmussen et al., 2000). However, in contrast to the present study, they reported reduced HPA axis activity; characterized by reduced pituitary POMC mRNA expression, reduced in

adrenal weight and an 'inferred' reduction in corticosterone levels (describing and 'trend' rather than statistical significance). These results were reported following 3 weeks abstinence from a 50 day liquid diet schedule in rats. Increased free corticosterone levels during acute withdrawal were also reported in their study, but they did not report on any measurement of free corticosterone following long-term abstinence. This would have enabled a more accurate comparison between the two studies. They found no statistically significant change in total corticosterone levels after abstinence (although they reported a trend) concurring with the present study. They concluded that HPA axis activity is reduced during abstinence, and reduced corticosterone levels, contributing to negative reinforcement, may be an important factor in relapse. In the present study it has been demonstrated that brain corticosterone levels can be different even when total plasma levels do not differ, perhaps their study may have benefited from measurement of brain corticosterone levels.

Future work

Although testing the activity of more doses of corticosterone, aldosterone and their respective antagonists may be useful in determining all the information about the influence of adrenal steroids on locomotor activity, further work on corticosterone administration and locomotor activity after liquid diet will probably not provide useful information about the relationship between corticosterone and dependence

Increased anxiety is reported in humans during long-term abstinence, therefore assessments of anxiety–like behaviour in mice during abstinence may be useful. The influence of different doses of corticosterone, and the MR antagonist spironolactone would provide a more complete picture than just the

administration of RU 38486 did on long-term alterations in corticosterone responsiveness after chronic alcohol treatment.

In situ-hybridization and immunohistochemical analysis of corticosterone, CRF, ACTH and their respective receptors as well as the measurement CRF and ACTH levels could provide a more detailed picture of the alterations in the HPA axis during long-term withdrawal. Measurement of these parameters at different time points during alcohol treatment and the subsequent withdrawal would also aid the understanding of the development of these changes.

Chapter seven

Dopamine receptors and abstinence

Introduction

Dopamine

In this chapter the focus of the work is on the long term effects of alcohol on the dopaminergic system. Dopamine is the major catecholamine in the central nervous system. It is involved in the regulation of a variety of functions, including locomotor activity, emotion and neuroendocrine secretion as well as reward and addiction. The introduction of molecular cloning procedures resulted in a major shift in the understanding of the dopaminergic receptor system. Although five dopamine receptor subtypes have been cloned (Sokoloff, 1995), the D1/D2 classification is still relevant (Jaber et al., 1996). D1 and D5 receptors are classified as D1-like, because they share a high sequence homology, stimulate adenylate cyclase and share classical D1 receptor pharmacology. The D2, D3 and D4 receptors are considered to be D2-like, mainly because of their homology and pharmacology (Jaber et al., 1996).

The D1 receptor is the most widespread dopamine receptor and is expressed at higher levels than any of the other dopamine receptors (Dearry et al., 1990). D1 and D2 receptors are found mainly in the striatum, nucleus accumbens, and the olfactory tubercle. Dopaminergic neuron cell bodies are localised mainly in the substantia nigra pars compata, the ventral tegmental area and the hypothalamus. They define three main dopaminergic pathways the nigrostriatal, the mesolimbic, and the tuberoinfundibular. The mesolimbic

dopaminergic system consists of the cell bodies in the VTA which project to the limbic areas, such as the nucleus accumbens.

The mesolimbic dopaminergic system and reward

The mesolimbic system can be implicated in the reinforcing properties of most drugs of abuse (Wise et al.,1990; Koob 1992; Self & Nestler 1995). Rodents will self-administer alcohol (Gatto et al., 1994) and morphine (David and Cazala, 1994), directly into the VTA. Local injection in the VTA of opiates, amphetamine, cocaine or nicotine produced conditioned place preference (Bozarth and Wise, 1981). Different drugs of abuse, including opiates and psychostimulants (Nestler, 1992) and alcohol (Ortiz, 1995), have been reported to produce similar neurochemical changes in the mesolimbic dopamine pathway both during administration and acute withdrawal, despite the different initial target sites. Acute alcohol administration has been shown to increase the firing of VTA neurones both in vivo (Gessa et al., 1985) and in vitro (Brodie et al., 1991). Administration of alcohol into the VTA enhances dopamine release in the nucleus accumbens (Di Chiara & Imperato, 1985). Reductions in extracellular dopamine have been detected in the ventral striatum during withdrawal from alcohol, these reduced levels are seen 10 hours after withdrawal from chronic alcohol treatment but return to normal levels after 24 hours of withdrawal (Rossetti et al., 1992).

In many cases there is evidence that the drug-induced changes in dopamine levels are correlated to changes in levels of dopaminergic metabolites in the limbic areas. The main pathways for the metabolism of dopamine involve the enzymes catechol-O-methyl transferase and monoamine oxidase, which produce dihydoxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA).

Reggiani (1980) demonstrated increased levels of DOPAC in the striatum following acute administration of alcohol, and with chronic administration a tolerance to this effect was observed. Systemic administration of both dopamine agonists and antagonists appears to decrease alcohol reinforced responding (Pfeiffer et al 1988, Samson et al., 1992) but appeared to do so by different mechanisms; agonists altered the initial high rate of responding whereas antagonists caused early termination of the drinking period.

Long term changes after chronic alcohol treatment

Despite the knowledge of the acute mechanisms of the action of alcohol, much less is known about the chronic adaptations that alcohol produces in the brain after prolonged administration and long-term abstinence. However, it has been demonstrated that chronic alcohol consumption causes long term behavioural changes that are evident when other drugs of dependence are administered. Increased sensitisation to the locomotor effects of amphetamine, cocaine (Manley and Little, 1997) and of nicotine (Watson and Little, 1999) was first shown. These changes were seen when the psychostimulants were first given 6 days and even two months after cessation of chronic alcohol intake, indicating that prolonged changes are caused by the alcohol. In addition to these behavioural studies, electrophysiological experiments suggest progressive changes following withdrawal from chronic alcohol consumption. After the same alcohol treatment schedule (23 day liquid diet schedule) the firing rate of dopamine-sensitive ventral tegmental neurones in vitro was very greatly decreased at 24h and at 6 days after cessation of alcohol intake, but had returned to normal levels by the 2 month interval (Bailey et al., 1998; Bailey, 1998).

The decreased VTA firing rate seen in studies performed this laboratory in vitro during and upto 72 hours after alcohol withdrawal has also been reported in rats in vivo (Diana et al., 1996). Liljequist (1978) found increased locomotor stimulant actions of dopamine, injected into the nucleus accumbens, up to 4 weeks after withdrawal from chronic ethanol intake. Increased acute locomotor stimulation by cocaine and increased striatal dopamine transporter density was found by Itzak and Martin (1999) after repeated injections of alcohol. Nestby et al (1997), also using alcohol injection, saw increased acute locomotor stimulation by morphine, but not amphetamine, and increased dopamine release from the caudate putamen in vitro, 3 weeks after cessation of alcohol treatment. Voluntary alcohol drinking increased dopamine release, and increased dopamine D1-receptor stimulated adenyl cyclase activity after 3 weeks abstinence (Nestby et al., 1997). May (1982) found increased agonist affinity for D1 receptors 7 months after withdrawal from a alcohol treatment period of 36 weeks. An investigation of monoamine and metabolite concentrations showed increased dopamine metabolite to dopamine ratios at the 2 month after the cessation of chronic alcohol administration (Bailey et al., 2000). There were no changes after chronic alcohol treatment in the release of dopamine from striatal or cerebrocortical slices in vitro (Bailey et al., 1998; Manley, 1997), suggesting that the release process at the terminal areas is unaltered by alcohol treatment and abstinence.

Aims

The hypothesis to be tested is that chronic alcohol consumption causes prolonged alterations in mesolimbic dopamine function. These changes may be responsible for the increased rewarding effects and increased sensitisation seen

for other drugs of dependence during the abstinence period after chronic alcohol intake. Potential alterations (measuring the affinity constant, Kd, and maximal binding, Bmax) in dopaminergic receptors D1and D2 function were investigated using radioligand binding following six day withdrawal (seen Section Two, introduction) from 23 day alcohol administration via liquid diet.

Methods

Under reverse phase lighting conditions, male TO mice (inhouse bred) 25-35g, underwent a 23 day liquid diet treatment, as previously described (Chapter Six). Control animals were given isocalorific liquid diet containing no alcohol. Upon completion of the diet treatments at 10 a.m., the diet was replaced with standard laboratory chow. The animals were humanely killed by cervical dislocation six days after the termination of the diet treatment. The brains were dissected over ice and the cerebral cortex and striatum removed. These samples were rapidly frozen (isopentane /acetone-dry ice freezing trap –40°C) and stored in the freezer until required for the binding assays (n=6 per group).

Radiochemicals

^{3}H SCH23390 (70mCi/mol) and ^{3}Hspiperone (65mCi/mol) were both purchased form Amersham, U.K.

D1 Homogenate binding

D1 receptor binding was performed using a the previously described procedure (Dewar et al., 1990). After thawing, the samples were homogenised (Ultra Turrax, IKA Labortechnik, Fisher Scientific) in 20 volumes (w/v) of modified Tris buffer pH 7.4 (50 mm TrisHCl, 20 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaC1_2$). The suspension was then centrifuged for 30mins x16,000 g at 4°C. The supernatant was discarded, and the pellet resuspended and centrifuged under the same conditions for a further 30mins. The pellet was then resuspended in buffer to give a final tissue concentration of 10mg original wet weight in 1ml of buffer. The membrane preparation (250µl) was incubated at room temperature for two hours with [^{3}H]SCH23390 (250µl, six concentrations, 150pM-4nM). Non-

specific binding was defined by parallel incubations of 100 mM butaclamol (ina volume of 500µl buffer), for the total binding 500µl of buffer was added. The total volume per assay tube was 1ml. The assay was terminated by rapid filtration through GFC filters (presoaked in 0.1% polyethyleneimine) followed by three 3ml washes of cold buffer. The filters were transferred to scintillation vials, scintillation fluid was added and the radioactivity counted. Protein content was assessed by the Lowry method (see below). Saturation curves were plotted using Graphpad Prism data analysis software.

D2 homogenate binding

A standard binding assay, which had been previously described by Dewar et al., (1990) was used to measure D2 receptor binding. After thawing the samples were homogenised in 20 volumes (w/v) of modified Tris buffer pH 7.4 (50 mm TrisHCl, 20mM NaCl, 1mM $MgCl_2$, 2mM $CaC1_2$). The suspension was then centrifuged for 30mins x16,000 g at 4°C. The supernatant was discarded, and the pellet resuspended and centrifuged under the same conditions for a further 30mins. The pellet was then resuspended in buffer to give a final tissue concentration of 10mg original weight in 1ml of buffer. The membrane preparation was incubated at room temperature for two hours with [^{3}H]spiperone (250µl, six concentrations, 0.25nM-6nM) and 1mM ketanserin (250µl)to block 5HT receptor binding. Non-specific binding was defined by parallel incubations of 100 mM sulpiride (250µl). The assay was terminated by rapid filtration through GFC filters (presoaked in 0.1% polyethyleneimine) followed by three 3ml washes of cold buffer. The filters were transferred to scintillation vials, scintillation fluid was added and the radioactivity counted. Protein content was

assessed by the Lowry method (see below). Saturation curves were plotted using Graphpad Prism data analysis.

Protein assay

The quantity of protein in each sample was assayed using a protein assay kit (Sigma) based Peterson's modification of the Lowry method (Lowry et al., 1951; Peterson et al, 1977). The homogenates were diluted 1 in 100. To 100µl samples of the dilution 900µl of Lowry reagent was added. This reagent consists of 0.1% copper sulphate, 0.2% potassium tartrate, 10% sodium carbonate). The mixture was transferred to a 2.5ml cuvette and allowed to stand at room temperature (23°C) for 30 minutes. Folin and Ciocalteu's reagent was then added and the cuvette was vortexed for 5-10 seconds, after which it was allowed to stand for 30 minutes. The absorbance at 740nm was measured using a spectrophotometer. The photometer was first calibrated using a blank cuvette that contained only distilled water. The protein levels were quantified (after suitable dilution of the sample – 1/100) by comparison with a standard curve created from data using solutions of BSA at 50, 100, 200, 300, and 400µg/ml

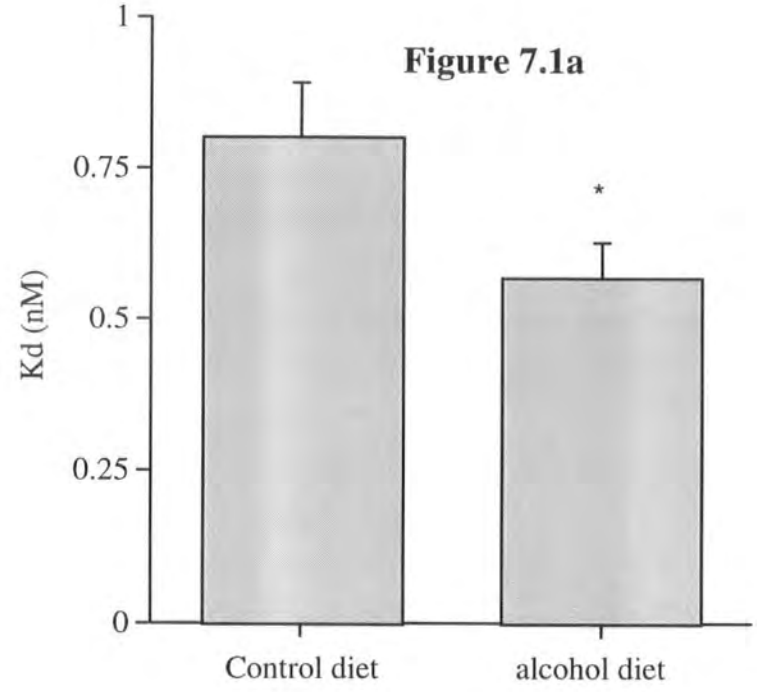
Statistical analysis

The Bmax and Kd values of control and alcohol treated mice were compared for each brain region using an unpaired Student's t-test. This test was chosen as it is suitable for comparing two groups of parametric data.
The Bmax and Kd values were measured from saturation curves generated by Graphpad prism. The data was fitted, by this program, to the line of best fit using the one binding site curve as multiple binding site curves were rejected as there was not a sufficient fit.

Results

In both studies the Bmax values are expressed as mean fmol/mg protein ±SEM And the Kd values are expressed as mean nM ±SEM. In the striatum maximum binding (Bmax) of [^{3}H]SCH23390 was unaltered by diet treatment (Figure 7.1b). The dissociation constant (Kd) of D1 receptors in the striatum was lower in the alcohol diet treated mice compared with the control diet treated mice ($P<0.05$) (Figure 7.1a) [^{3}H]SCH23390 binding in the cortex of control diet and alcohol diet mice showed no change in Bmax (Figure 7.2a) between the two groups. The affinity of D1 receptors in the cortex was not affected by diet treatment (Figure 7.2b). D1 receptor binding was higher in the striatum than in the cortex irrespective of the diet treatment. The binding in the striatum for D1 receptors was less variable than cortical binding.

Both the Kd (Figure 7.3) and Bmax (Figure 7.4) of D2 receptors in the cortex and the striatum were unaffected by diet treatment.



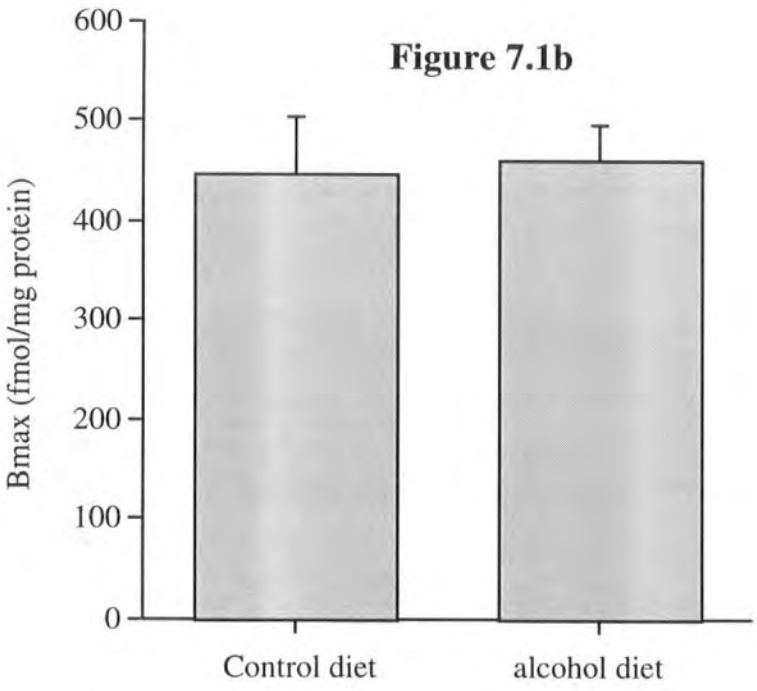
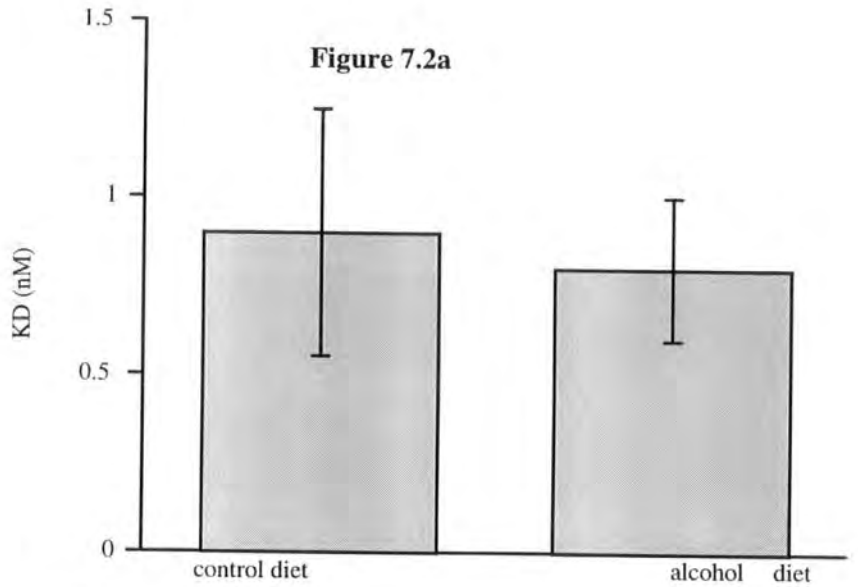


Fig 7.1: *Six days after withdrawal from the alcohol diet (n-6 per group). The D1-like receptor ligand ^{3}H-SCH23390 showed a significantly (*P< 0.002) higher affinity for striatal membranes (Figure 7.1a), no differences in Bmax were seen in the striatum (Figure 7.1b).*



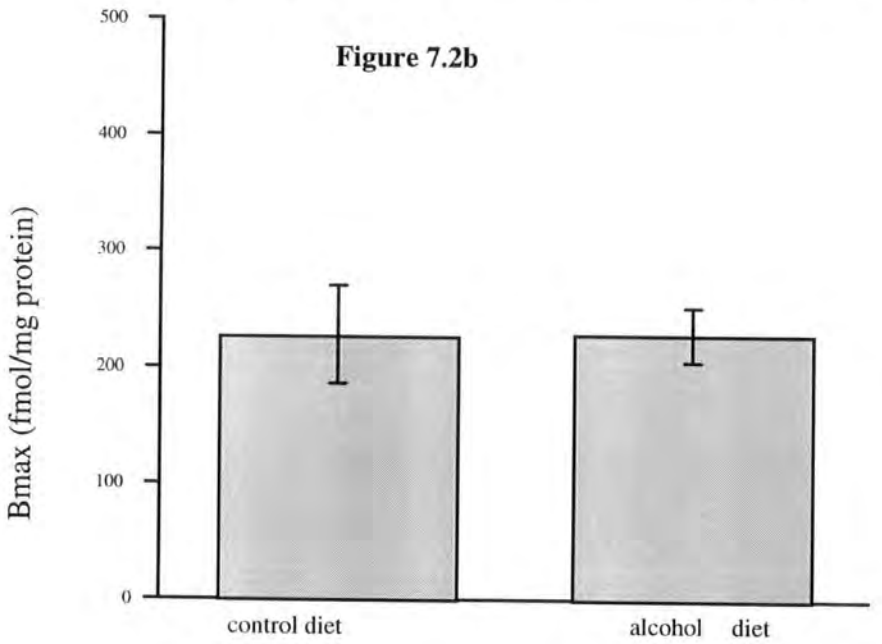


Figure 7.2 *Six days after withdrawal from the alcohol diet, no differences were seen in either the Kd (Figure 7.2a) or Bmax (figure 7.2b) values for binding of the D1-like receptor in the cortex (n=6 per group).*

Figure 7.2.1a

Figure 7.2.1b

Figure 7.2.1c

Figure 7.2.1d

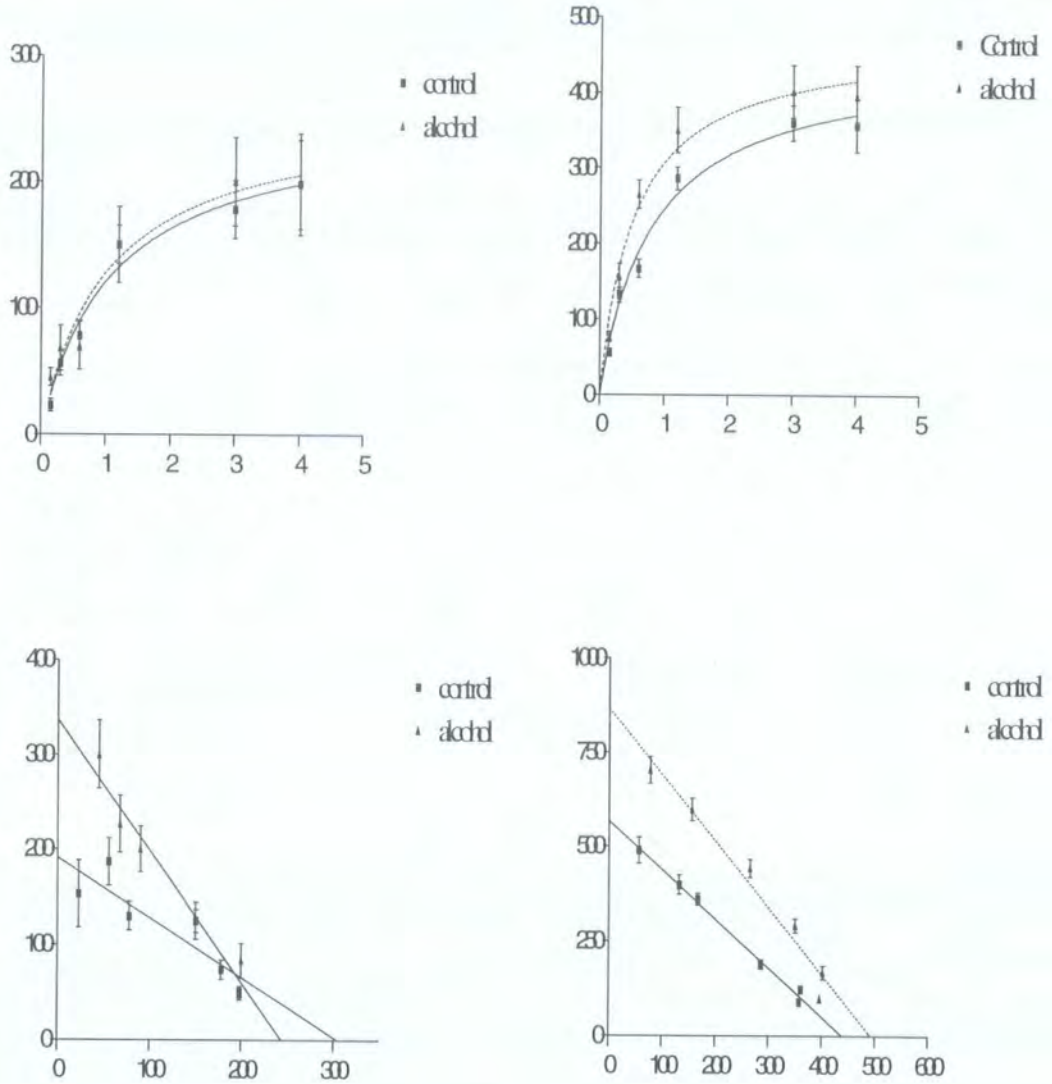


Figure 7.1.2 *To binding of [^{3}H]SCH23990 in alcohol and control diet treated mice.* **Figure 7.1.2.a** *shows the saturation binding curve of [^{3}H]SCH23990 to cortical membranes* ***and*** **Figure 7.1.2.b** *striatal binding. Scatchard plots (Scatchard, 1949) of the data are shown in* **Figure 7.1.2.c** *(cortex) and* **Figure 7.1.2.a** *(the striatum).. The Scatchard analysis gives a higher estimation of the Bmax values than the saturation curve. It should also be noted that additional saturation experiments with more concentration points and inhibition curves as well as autoradiography will provide greater detail and information on the 'small' but significant change in Kd recorded in these studies.*

Figure 7.3

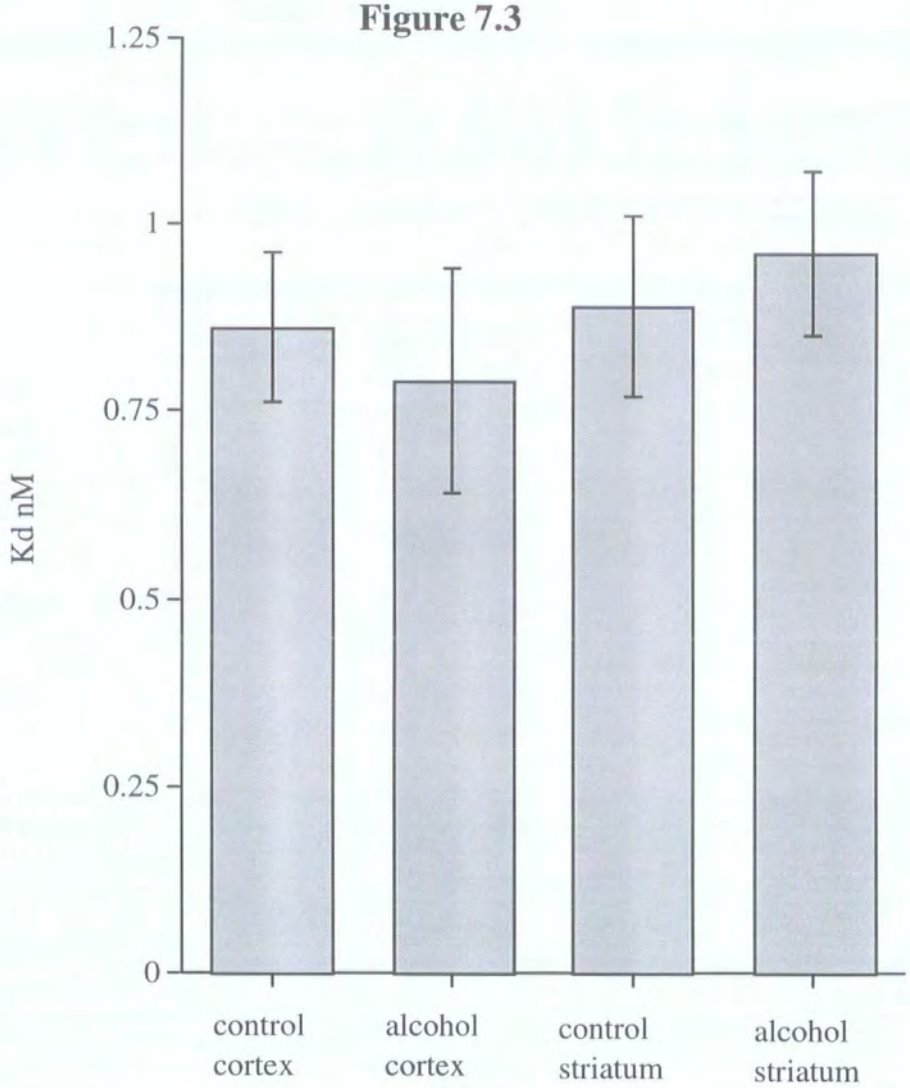


Figure 7.3 *Six days after withdrawal from the alcohol diet, no differences were seen in Kd values for the binding of [^{3}H] spiperone to D2-like receptors in the cortex or the striatum.*

Figure 7.4

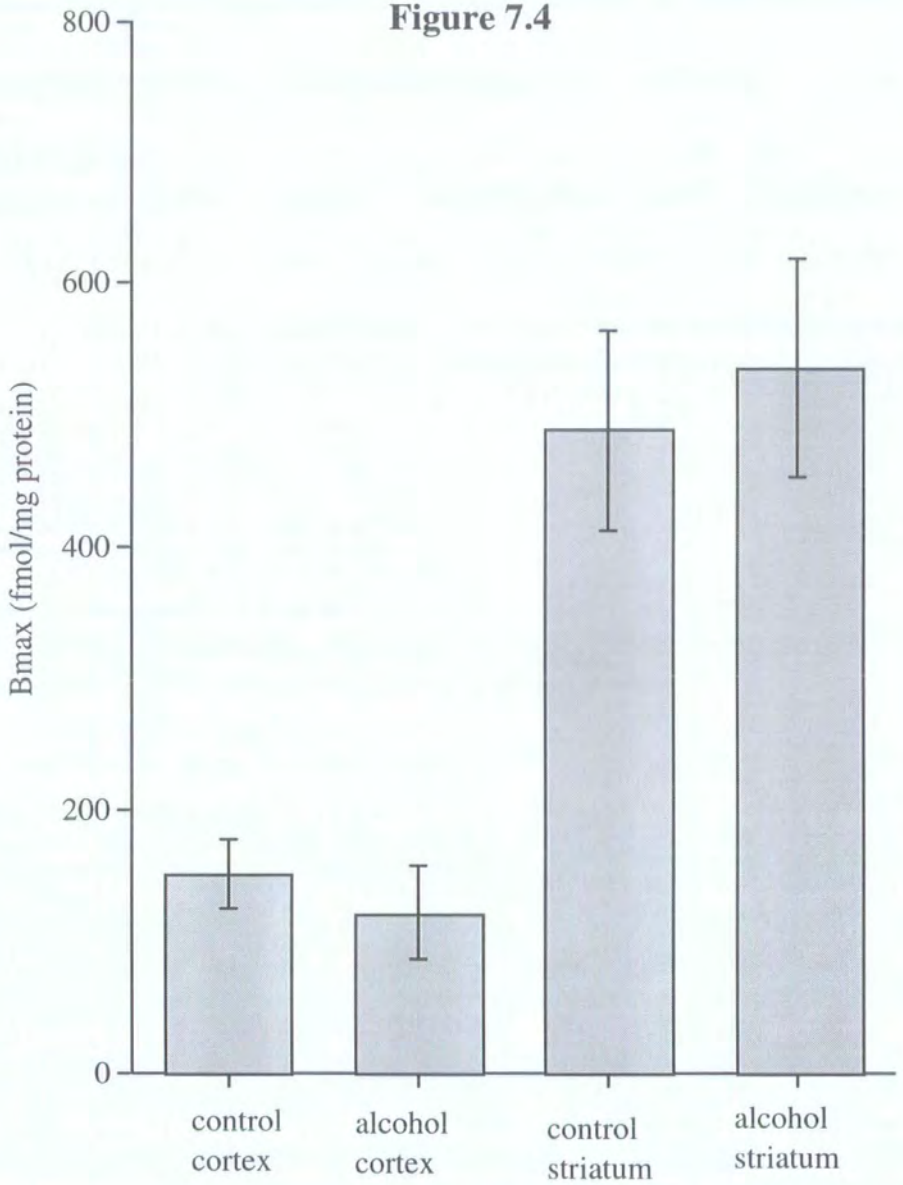


Figure 7.4 *Six days after withdrawal from the alcohol diet, no changes in Bmax of [^{3}H] spiperone binding to D2-like receptors were seen in either the cortex or the striatum.*

Figure 7.5a

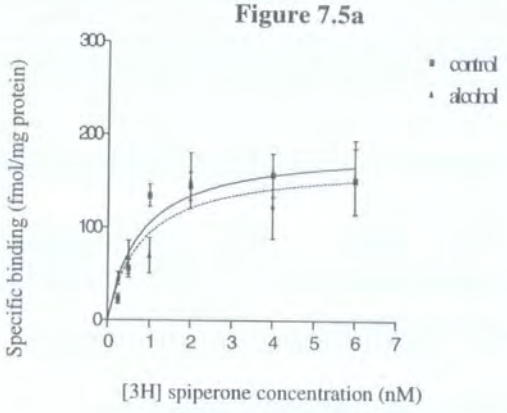


Figure 7.5b

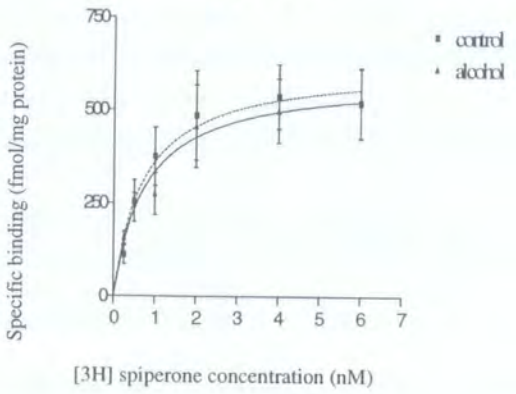


Figure 7.5c

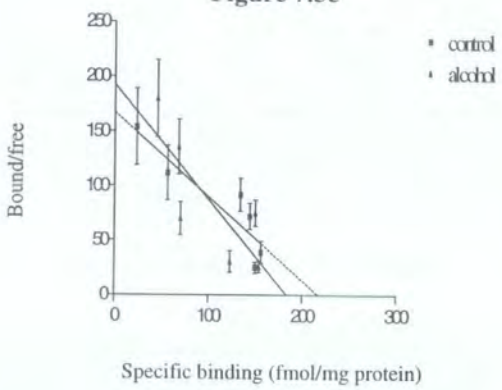


Figure 7.5d

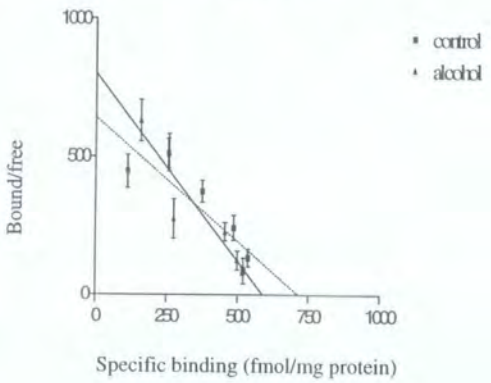


Figure 7.5 *To binding of [^{3}H]spiperone in alcohol and control diet treated mice (Kd and Bmax values are presented in Figures 7.1a and 7.1)b.* **Figure 7.5a** *shows the saturation binding curve of [^{3}H]spiperone to cortical membranes and* **Figure 7.5b** *shows the striatal binding. Scatchard plots (Scatchard, 1949) of the data are shown in* **Figure 7.5c** *(cortex) and* **Figure 7.5d** *(the striatum).*

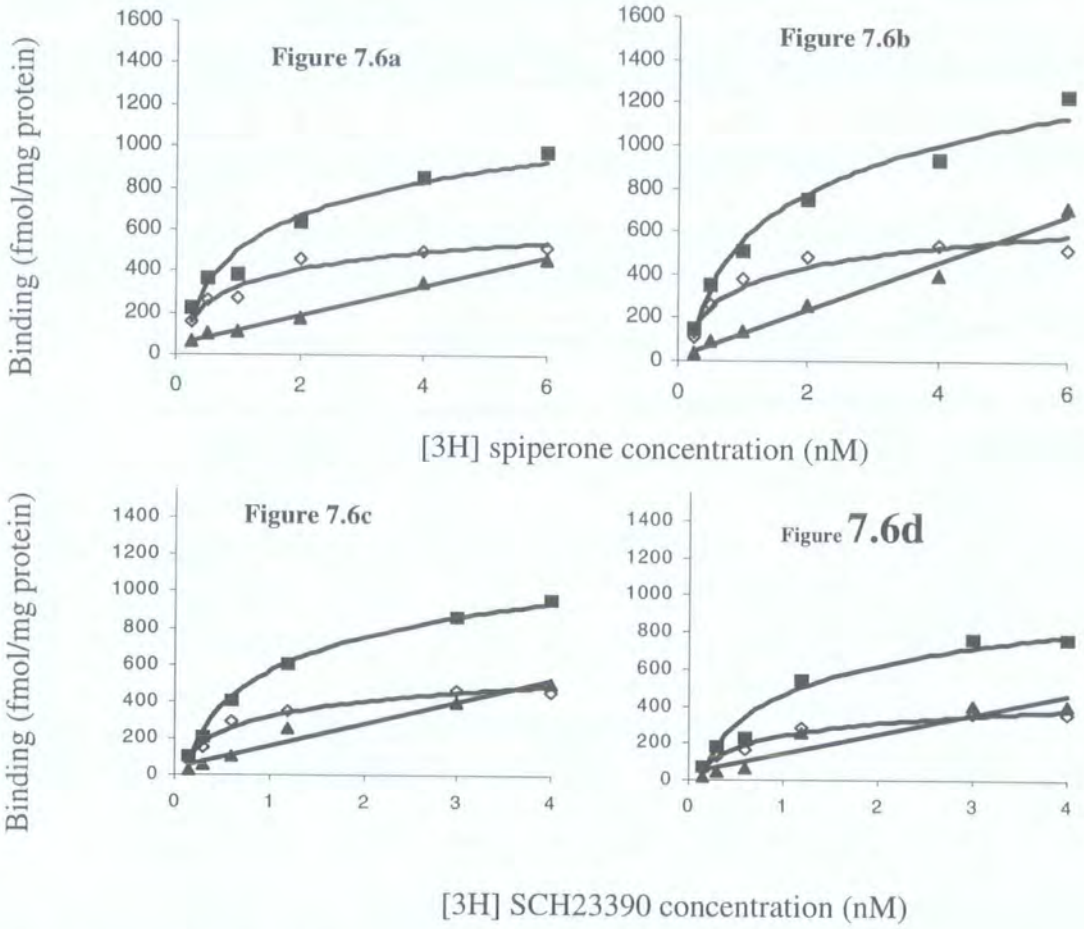


Figure 7.6 *Samples of the total (■), specific (◇) and non-specific (▲) binding of [^{3}H] SCH23390 and [^{3}H] spiperone.* **Figure 7.6a** *[^{3}H] spiperone binding to striatal membranes from control diet treated mice* **Figure 7. b** *[^{3}H] spiperone binding to striatal membranes from alcohol diet treated mice.* **Figure 7.6c** *[^{3}H] SCH23990 binding to striatal membranes from alcohol diet treated mice* **Figure 7.6d** *[^{3}H] SCH23990 binding to striatal membranes from control diet treated mice.*

Discussion

Increased affinity

The ligand binding studies on D1-like receptors and D2-like receptors indicated that, after the chronic alcohol intake, there was an increased receptor affinity for the D1 receptor subtype in the striatum, revealed as a decrease in Kd values. There were no significant changes in Bmax of Kd in the cortex. There were no significant changes in Bmax in this area suggesting no change in receptor density. No changes in either measurement were observed in cortex. D2-like receptor binding showed no changes in affinity nor receptor density in any of the brain areas studied.

The increase in affinity seen in the present study differs form the affinity changes reported by May. The increase in affinity demonstrated in the present study was identified by a reduction in Kd in saturation binding studies using SCH23390. The alteration reported by May was an decrease in Ki measured using labelled SCH23390 and unlabeled dopamine as the competitor. The mechanism responsible for the increase in affinity may be different. In the Introduction the existence of multiple dopamine receptor subtypes was described. The D1-like receptor consists of two distinct receptor types the D1 and the D5 receptor (sometimes described as the D1a and D1b receptor subtypes) (Sunahara et al., 1991). Both these receptors have the same affinity for SCH23390, but the D5 receptor has been reported to show a higher affinity for dopamine (Sunahara et al., 1991). May (1982) speculated that an increase in the number of D5 receptors relative to 'D1' receptors (high dopamine affinity D1-like receptors) might have been responsible for the observed increase in affinity. This is not a possible explanation for the results in the present study as it is not possible to

distinguish D1-like receptors on the basis of their binding to SCH23390. A conformational change in the receptor itself or linked to the receptors' signal transduction mechanism, would be the most likely mechanism for the observed change in affinity.

A possible mechanism for relapse

The observed increase in affinity is consistent with the increased locomotor stimulant effects of nicotine (Watson & Little 1999), and also of psychostimulant drugs (Manley & Little 1997). Sensitisation, seen after repeated administration of many abused drugs, has been implicated in drug dependence and relapse, as the phenomenon lasts for many months, possibly permanently, and it develops to the rewarding effects of the drugs (Robinson and Berridge, 1993; Lett, 1989).

A progressive series of changes in neuronal function appears to take place during the sensitisation process and further changes occur after cessation of repeated administration of psychostimulant drugs. For 3-4 days after repeated administration of amphetamine or cocaine, dopamine autoreceptors in the VTA show subsensitivity. At a later stage, there are transient increases in excitatory amino acid function (White, 1996). The results are also consistent with an increased D1-like receptor stimulation of cAMP production several weeks after cessation of chronic ethanol treatment (Nestby, 1997) and the increase in affinity of D1 receptors reported by May (1982).

The increase in affinity of D1-like receptors, which are primarily postsynaptic in location, would compensate for the decreased firing seen in the VTA following abstinence from chronic alcohol administration (Bailey et al., 1997), so that overall synaptic efficiency at these sites would continue, and the

animals appear behaviourally overtly normal. Factors that affect relapse e.g. stress or a small priming dose of alcohol or another drug of abuse would enhance the resultant effects of released dopamine acting on the altered D1 receptors leading to an increase in the rewarding effects of alcohol.

There is considerable evidence that sensitisation to psychostimulants is mediated via the mesolimbic system (Sorg and Ulibarri, 1995; Henry and White, 1992; Kita et al., 1992) and that D1 receptors in particular are linked to sensitisation. It has been shown that D1 receptor activation is necessary for the induction of sensitisation by amphetamine in the VTA (Vezina, 1996). D1 receptor antagonists have been shown to prevent the facilitation of amphetamine self-administration induced by prior exposure to the drug (Pierre & Vezina, 1998). This would suggest that the changes in D1 receptor affinity may predispose alcohol-dependent individuals to abuse of other drugs.

D2-like receptors and chronic alcohol

Although no changes in D2 receptors were found other researchers have reported changes after alcohol treatment. Chronic alcohol, administered as sole fluid, has been shown to increase the binding affinity of [^{3}H] spiroperone during acute withdrawal; 24 hours after cessation of treatment, a down regulation of D2 receptors occurs, with an upregulation apparent at five days in the caudate putamen, nucleus accumbens and olfactory tubercle (Rommelspacher et al 1992). Investigating earlier time points , Reggiani et al (1980) demonstrated an increase in ^{3}H spiroperidol binding in the striatum, as measured three hours after chronic alcohol treatment. However, Hruska et al (1988) found no change in D2 receptors density in the rat striatum at 24 hour withdrawal, whereas Muller et al (1980) demonstrated a decrease in D2 receptors in the nucleus accumbens but not

striatal areas. It appears from these reports and from the results of the present study that possible changes in D2 receptors do not persist beyond the acute withdrawal phase. Modell et al (1992) have reported that the dopamine antagonist haloperidol can reduce craving for alcohol in alcoholics withdrawn from alcohol for 2 to 14 weeks, although the drug had side-effects (sedation) which may have contributed to the results.

Relapse and dopamine receptors.

Investigations into the dopaminergic system and relapse has generally focused on the influence of the dopamine on relapse to cocaine. Reinstatement of cocaine administration in operant responding situations has been used to investigate the effect of dopamine D1 and D2 like receptor agonists and antagonists (Self et al., 1996). The researchers tested the ability of compounds that selectively stimulate specific dopamine receptors to reinstate self-administration of cocaine in animals who had stopped seeking this drug. In this study, rats were allowed to self-administer intravenous cocaine for 2 hours and then saline was substituted for 2 hours during which time the self-administration behaviour progressively diminished (to extinction). The researchers found that when a D2-like agonist was administered to these animals, a dramatic increase in cocaine-seeking behaviour was observed, while administration of a D1-like agonist had no effect. They further tested whether the D1-like or D2-like agonists could block the effects of cocaine on reinitiating cocaine use. In these rats, providing a small priming dose of cocaine causes an increase in cocaine-seeking behaviour. While pretreatment with D2-like agonists in these animals caused dramatic potentiation of cocaine's priming effects, pretreatment with D1-like agonists completely prevented cocaine's ability to reinstate cocaine use. These

results lead to the proposal that cocaine craving and relapse can be provoked by activating D2-like receptors, but activating D1-like receptors actually prevented cocaine-seeking behaviour. Similar results and conclusions have been proposed by other researchers (Khroyan et al., 2000).

The increased D1 receptor affinity and the lack of change in D2 receptors demonstrated in the present study would appear to contradict these findings. There are a number of possible explanations, the first being that the mechanism behind relapse in alcohol dependence differs from the mechanism for relapse behind cocaine dependence. Another explanation may be that two hours is probably not sufficient time for long-term neurochemical changes to occur. The changes observed in the current study developed after 23 days of diet treatment and 6 days of abstinence, it would be doubtful whether these changes would be apparent after alcohol treatment for one day. The effects of D1 and D2 drugs seen in the cocaine studies are as likely to be a consequence of selective receptor desensitisation or tolerance as they are to be a model of relapse. The changes of receptor affinity measured in the present study provide evidence for long-term changes following chronic alcohol treatment and withdrawal and could provide a start in the understanding of the neurochemical basis of relapse.

Further work.

Receptor autoradiography would give better spatial resolution of the changes, and perhaps isolate them to a particular region of the striatum. The autoradiography should not be confined to the D1 receptor, the other dopamine receptors and the dopamine reuptake receptor should be investigated. The time points at which the experiments are done should also be extended to include measurements during the alcohol diet treatment and at more time intervals after the treatment. Binding during the acute phase of withdrawal and at time points after the six days, up to and including 2 months after the cessation of the treatment should be performed. The increase in responsiveness to psychostimulants reported by Manley and Little (1997) was apparent up to 2 months after the cessation of the diet treatment.

Experiments should be performed to ensure that the changes are not an artefact of the particular method of alcohol administration, e.g. inhalation chambers, or alcohol administered as sole fluid to a high alcohol preferring strain of rodent. Further work could also include investigations into other CNS receptor types. It would be naïve to assume that a change in D1 receptor affinity is the only measurable long-term change induced by chronic alcohol administration. These investigations could include glutamate receptors, 5HT receptors, voltage activated calcium channels and opiate receptors. This list is not exhaustive as alcohol is known to affect the functioning of numerous CNS systems.

Chapter Eight

General Discussion

Brain corticosterone levels

This thesis describes a number of novel and important findings. Aside from the general theme of the thesis (alcohol dependence) a new methodology was developed. A method for measuring brain corticosterone levels could prove to be valuable tool in research. McEwen (1998) have described the relationship between corticosteroids and the 'ageing brain'. They have proposed that increased *circulating* corticosterone levels could produce hippocampal neuronal atrophy acting in concert with excitatory amino acids, measurement of the actual brain (hippocampal) corticosterone levels could be of benefit. A long list of research that could utilise this methodology might distract from the main theme of the Thesis.

Summary

Experiments that were performed for this thesis showed that the corticosterone synthesis inhibitor metyrapone, and ACTH $_{4\text{-}10}$ reduced the alcohol preference of high alcohol preferring mice C57. However, ACTH $_{4\text{-}10}$ increased preference in low preferring mice as did the CRF antagonist, α-helical CRF. These results demonstrate the need to consider the role of the whole HPA axis on alcohol consumption rather than one of its components. This work has provided important information on the role of both of these peptides in alcohol consumption as well as avenues for future research.

Vehicle injections, whether saline or tween, increased alcohol preference in low alcohol preferring mice and these injections were shown to increase free

circulating, and hippocampal, levels of corticosterone. The findings in these experiments are important in terms of general research. The effects of vehicle injections are not often considered in experimental work, and these studies have demonstrated clear behavioural changes induced by saline injections and by the insertion of a needle without any fluid being injected.

Work performed in this study also demonstrated long-term changes in blood and brain corticosterone levels during abstinence. Increased receptor D1 receptor affinity during abstinence was also shown. These long-term changes provide important information about the neuronal processes that may determine the propensity of alcoholics to relapse.

Dependence and relapse

A mechanism by which corticosterone's influence on drug-dependence is exerted has been proposed by Piazza (1996). This proposal focuses on the interaction between the dopaminergic system and the effects of corticosterone on this system. The two systems are known to interact, corticosterone enhances dopamine release both in vivo (Rouge-Pont et al., 1998) and in vitro (Rouge-Pont et al., 1999). Concentrations of glucocorticoids can determine the level of dopamine release in the nucleus accumbens (Piazza et al., 1998) and increase excitatory amino acid activation in the VTA (Cho & Little, 1999).

In basal conditions, sensitivity to the reinforcing effects of drugs of abuse are low, as glucocorticoid secretion and dopamine release are low. An acute stress increases corticosterone secretion, which enhances the release of mesolimbic dopamine, and so results in the reinforcing effects of drugs of abuse. This can lead to an increase in drug self administration. Activation of negative feedback by corticosterone returns the system to basal levels . The binding of

corticosterone to hippocampal corticosteroid receptors is a key step in the activation of the negative feedback. The repeated increase in the glucocorticoid induced by repetitive stress will progressively impair negative feedback and this will probably decrease the number of central glucocorticoid receptors in the hippocampus. An impairment of negative feedback will result in extended secretion of these hormones and elevate dopamine in the nucleus accumbens. Piazza proposed that these changes can in turn determine a long-lasting increase in the sensitivity to the reinforcing effects of drugs of abuse. Both in the increase in corticosterone levels and the increased D1 receptor affinity, shown in the present study could influence relapse. The corticosterone, by the impairment of negative feedback causing elevated dopamine in the nucleus accumbens. The effects of this elevated dopamine would be amplified because of the increased receptor affinity.

However, the role of the hypothalamic and pituitary peptides suggest this model may be over simplified. The increased HPA axis activity and the disrupted negative feedback in addition to higher circulating levels of corticosterone will also produce higher levels of both ACTH and CRF. These may decrease rather than increase alcohol consumption. The relative importance of corticosterone, ACTH and CRF needs to be assessed. The present study has demonstrated the profound actions of all three on alcohol consumption. The next stage of work should be to further understand combined influence of them rather than the isolated influence. The regulation of the HPA is such that if the levels of one component changes there is a reactive change in the others.

Final comments

The importance of the problem of alcohol dependence for society cannot be over-emphasised, some of the array of problems (medical, social and economic) were outlined in the introduction to this thesis. The treatment of alcohol dependence by drugs has developed recently, and the reports on the effectiveness of the new dependence drugs such as acamprosate (Lhuintre et al., 1985, 1990; Whitworth et al., 1996) and naltrexone (O'Malley et al., 1992; Volpicelli et al., 1992) are encouraging, but even with these therapies relapse rates are still high, 23% for acamprosate and 17% for naltrexone measured after12 week, the relapse rates increase further with time despite the medication (Swift, 1999). The work performed for this thesis has produced information towards the understanding of the neuronal basis of alcohol dependence, this may assist in the search for more effective pharmacotherapies for alcohol dependence.

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