A new rat model for investigating the effects of alcohol on the adolescent brain: Acute and long-term effects

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Abstract

Adolescence represents a time of unique vulnerability to improper brain maturation, due to continued development of some frontal regions of the brain and a propensity for risky behaviours including alcohol use. Recently, in New Zealand and overseas, increases in binge drinking during adolescence have necessitated further research into alcohol’s short- and long-term effects on the brain and behaviour. Important research using animal models has revealed gross changes in brain structure and function in response to large alcohol doses, however it is still not known what subtle changes, if any, may result from binge drinking at the levels that are seen in the majority of the population.

The current study aims to address this by using Long-Evans rats to model moderate- to high-levels of weekly binge drinking in human adolescents. Male and female rats were given 9.0 g/kg/day alcohol by intra-gastric gavage, every fourth day from PN28 to 48, resulting in a mean peak BEC on alcohol dosing days of 277 mg/dL.
Control animals were not given alcohol. The brains of the rats were investigated immediately following alcohol treatment (ages PN29, PN33, PN37, PN41, PN45 and PN49) for presence of apoptotic cell death and gliosis. A battery of behavioural tests was completed up to a year of post-natal age in order to investigate changes in brain function that persist in the long-term.

Histological survey throughout the cerebrum revealed no apoptosis, which could be consistent with an insult, after any of the 6 alcohol treatments. There were, however, subtle differences in astrocyte number in the rostral cingulate cortex, which require further study. Behavioural tests revealed subtle differences in performance between treatment/sex groups in key areas, despite a considerable period of abstinence. Of particular interest was an alcohol-induced reduction in working memory performance, during recognition of a novel object, that affected male but not female rats. Male, alcohol-treated rats also were less accurate at finding the platform in the initial stages of the MWM probe trial, as evidenced by fewer crossings of the previous platform location. This finding was not replicated in the female rats. Female rats that had been treated with alcohol during adolescence did, however, spent a significantly greater percentage of their path length in the outer zone.
of the MWM during the probe trial and this behaviour is normally indicative of a non-problem solving, panic response.
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Thanks to Ruth, her lab and the Anatomy Department for allowing this research to happen. Most importantly thanks to my friends and family for making me stick with it.
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<tbody>
<tr>
<td>ALAC</td>
<td>Alcohol Licensing Advisory Committee</td>
</tr>
<tr>
<td>AOI</td>
<td>Area(s) of Interest</td>
</tr>
<tr>
<td>AUD</td>
<td>Alcohol Use Disorder</td>
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<td>AUDIT</td>
<td>Alcohol Use Disorders Identification Test</td>
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<td>BEC</td>
<td>Blood Ethanol Concentration</td>
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<td>BIS</td>
<td>Behavioural Intoxication Scale</td>
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<tr>
<td>BPU</td>
<td>Behavioural Phenotyping Unit</td>
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<tr>
<td>BrdU</td>
<td>Brxodeoxyuridine</td>
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<tr>
<td>CA1</td>
<td>The CA1 sub-region of the hippocampus</td>
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<tr>
<td>COX-2</td>
<td>Cycloxygenase-2</td>
</tr>
<tr>
<td>dL</td>
<td>decilitre</td>
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<tr>
<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
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<tr>
<td>g</td>
<td>grams</td>
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<tr>
<td>G</td>
<td>Gestational day</td>
</tr>
<tr>
<td>g/kg</td>
<td>grams of alcohol per kilogram of animal weight</td>
</tr>
<tr>
<td>HIGS</td>
<td>Heat Inactivated Goat Serum</td>
</tr>
<tr>
<td>HTRU</td>
<td>Hercus Taieri Resource Unit</td>
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iNOS  Inducible Nitric Oxide Synthase
kg    kilogram
L     Litre
LTD   Long-term Depression
LTP   Long-term Potentiation
M     Molar
mg    milligram
Mg\(^{2+}\) Magnesium ions
MRI   Magnetic Resonance Imaging
MWM   Morris Water Maze
NMDA  N-methyl-D-aspartate
NR1-4 NMDA Receptor 1-4
PBS   Phosphate Buffered Saline
PET   Positron Emission Tomography
PN    Post-natal day
TLR-4 Toll-like Receptor 4
μm    Micrometre
mL    Millilitre
1 Introduction
Chapter One: Introduction

1.1 Introduction to Alcohol and Adolescence

Since the discovery of fermentation some 9000 years ago, humans have abused alcohol\(^1\) as a drug. In recent times this abuse has become prevalent in adolescents (de Bonnaire et al., 2004), possibly due to increased availability, decreased price, and marketing that is more than ever targeted at young drinkers. To understand why this is alarming, it is important to understand the important changes that are occurring, particularly in the brain, throughout adolescence.

Firstly, one must define adolescence. Adolescence is generally thought to be the period of life from puberty to adulthood. Puberty is easy to define biologically, however adulthood is often defined culturally, i.e. when the individual is considered, and takes on responsibilities befitting of, an adult. However, finishing biological development takes more time, especially the development of the brain (Lebel et al., 2008; Giedd et al., 2009; Giorgio et al., 2010).

\(^1\) Much of the literature refers (correctly) to the alcohol that is consumed recreationally as ‘ethanol.’ However, to the layperson it is ‘alcohol.’ So as not to contribute to the perceived distinction between the two, this thesis will refer to it as ‘alcohol’ unless the ethanol molecule (e.g. “ethanol concentration”) is being referred to.
1.2 The Adolescent Brain and its Development

Only with the development of higher resolution non-invasive imaging techniques that do not use radiation, have we begun to understand the changes that occur during adolescence to form an adult brain from a juvenile brain. Magnetic resonance imaging (MRI) has been an important technology that does not make use of ionising radiation but rather the detection of protons as they respond to a magnetic field. This is safer and has allowed the use of large sample sizes, healthy participants and longitudinal studies, all of which have broadened our understanding of normal adolescent development. Adolescence is a period of marked change in the brain, which incorporates changes at both the gross anatomical level down to the molecular level.

1.2.1 MRI Evidence: Grey Matter

It is now known that peak cerebral size relative to body weight occurs relatively early in development, the mean age being 10.5 years in girls and 14.5 in boys, and then decreases throughout the remainder of adolescence (Lenroot et al., 2007) to reach a smaller relative volume in adulthood. This is accompanied by an increase in volume of the lateral ventricles, which peaks in adulthood (Giedd,
The adolescent brain includes a larger volume of grey matter; that is the areas of brain that are dominated by the cell bodies of neurons and support cells. Generally speaking, the volume of grey matter increases until puberty and is followed by a decrease, beginning in the dorsal parietal regions rostrally through the frontal cortex before spreading caudally and laterally through the occipital and then temporal cortex, a pattern that follows behavioural maturation (Gogtay et al., 2004; Giorgio et al., 2010). The prefrontal cortex is the last region of the frontal cortex to develop during adolescence and it has a very specific role in higher order or executive functions. These functions arguably allow us to function as adults and it is important to note that the development of this key structure occurs throughout adolescent life, with maturity reached at the transition to adult brain structure and function.

Grey matter exhibits non-linear, inverted-U-shaped, volume changes from birth to maturity that differ between brain regions (Giedd, 2008). Frontal lobe grey matter increases during preadolescence, reaching peak levels at 10.5 years in males and 9.5 years in females before decreasing throughout adolescence (Giedd, 2008). These changes are similar to those in the parietal lobes, where peak occurs at 9 years in males and 7.5 years in females. In the temporal lobe, grey matter peaks at 11 years in males and 10 in
females with only a slight reduction over the course of adolescence (Toga et al., 2006; Giedd, 2008). It is considered that these changes in grey matter are not the result of changes in neuron number despite neurons being the principle component of grey matter, but reflect changes in the structure of the dendritic tree, the highly branched extensions of the polar neuron that receives action potentials from surrounding cells via synaptic contacts. The increase in grey matter to the peak volume is the result of dendritic growth and increased branching alongside synaptogenesis, while the decrease in grey matter that occurs during adolescence is a result of ‘pruning of the tree,’ the refinement of dendritic branching and synapses to form a final dendritic structure that subserves the complex circuitry of the mature brain (Huttenlocher, 1979).

1.2.2 MRI Evidence: White Matter

White matter, the other main component of brain tissue, also shows changes during adolescence (Colby et al., 2011). White matter generally consists of the long axons that neurons use to communicate both between brain regions and between distinct areas within a single region, as well as the supporting, non-neuronal cells that enclose and sustain them. Studies have revealed a linear increase in white matter of 12.4% on average between the ages of 4 and 22 years,
with males exhibiting a larger increase than females (Giedd et al., 1999). This rate is consistent across the four major lobes of the brain (Giedd, 2004). The total mid-sagittal area of the corpus callosum, the major white matter landmark of the brain, has been shown to increase markedly between the ages of 4 and 20 years (Giedd, 2008). A study of 90 subjects of varying ages that were scanned every 2 years showed growth of the corpus callosum until around 25 years of age (Pujol et al., 1993), with the greatest rate of increase occurring within the second decade. The increase in white matter is the result of myelination of nerve fibres, a process that increases the conduction speed of action potentials by up to 100 times (Giedd, 2004). Myelination occurs initially in the posterior structures of the brain before moving anteriorly (Giedd, 2004) and this process parallels the functional development of these structures (van der Knaap et al., 1991). The hippocampus has been shown to not alter in size significantly between the ages of 18 and 55 (Flaum et al., 1995) however it does undergo extensive myelination during the first two decades in humans (Benes et al., 1994).

1.2.3 Change in Brain Function During Adolescence

Maturation in the adolescent brain can also be studied on the basis of activity and how this changes between childhood,
adolescence and adulthood. Early studies of adolescent brain development used positron emission tomography (PET) which in these early studies measured the uptake of injected fluorodeoxyglucose in the body by tracing the gamma radiation that accumulated in cells as the glucose was metabolised (Phelps and Mazziotta, 1985). Uptake of the glucose analogue and the accumulation of radioactive marker reflects metabolic rate indicating the activity of that area of grey matter. A comparison of activity levels between adolescents and adults allows one to determine when different brain regions in the adolescent reach the same, or 'mature' level of metabolism, for a specific task, as in the adult. This method has been used to analyse the brains of children who, although they suffered transient neurological events, were selected as a reasonable representation of normal children (Chugani et al., 1987). The general trend was of increased metabolism in all brain areas beyond adult levels early in life, which began to drop at around the onset of puberty before reaching adult levels near the end of the second decade (Chugani et al., 1987). There were, however, differences observed between brain areas; the cerebral cortical areas showed the greatest metabolic increase, reaching 190 to 226% of adult values by the age of 8 years (Chugani et al., 1987). This means that the cerebral cortex also undergoes the greatest decline in activity to reach
maturity, where the redundancy of connections that develop in the child are pruned back and organised into a mature cortical network during adolescence (Huttenlocher, 1979). These data, although interesting, are based on a very small sample size, with the participants also taking daily anticonvulsant drugs. This raises questions as to the validity of the data for the ‘normal’ population but the findings do align with the general concept of an overabundance of grey matter at or immediately before puberty, which is refined to produce a more mature brain at around the end of the second decade (Chugani et al., 1987; Gogtay et al., 2004; Lenroot, 2007; Giedd, 2008; Giorgio et al., 2010). The use of ionising radiation is a limitation to carrying out functional studies so the development of functional MRI technologies has made a vast contribution to our understanding of maturational changes in the human brain.

Function, performance on particular behavioural tasks, changes during development and it is generally accepted to be accompanied by a change in brain structure, probably at the synaptic and molecular levels. Functional MRI has been used to look at the activation pattern of brain areas in response to three different executive tasks, and found that the level of activation undergoes a gradual maturation from childhood to mid-adulthood that is task-
specific, and that correlates with the key functional roles of different developmental ages (Rubia et al., 2006). Adults showed increased activation in frontostriatal networks, such as the right orbital and medial prefrontal cortex and caudate nucleus during the Go/No-Go task (a task that measures impulsivity by presenting a go/no-go cue after a ‘prepotent’ cue); right mesial and inferior prefrontal cortex, parietal lobe and putamen during the Switch task (a task that measures ability to adapt to switching between two opposing task conditions) and left dorsolateral and inferior frontotemporoparietal regions and putamen during the Simon task (a task that measures the effect of location of irrelevant stimulus on response accuracy and reaction time) (Rubia et al., 2006).

A maturational change has also been observed during spatial working memory tasks. Adulthood was associated with increased activity in bilateral portions of the superior frontal gyrus; left superior and middle frontal gyri; inferior aspects of the precuneus and angular gyrus; and in a cluster encompassing the right inferior parietal lobule, post central gyrus and insular cortex (Schweinsburg et al., 2005). These regions are involved in self-awareness (superior frontal gyrus), attention (precuneus and angular gyrus), complex abstract operations (inferior parietal lobule and angular gyrus), sensation, consciousness and other executive functions. These
increases in activity all reflect the development and refinement of the structures related to frontal cortical function and associated behaviours that become more developed to allow adult function. Childhood was associated with increased activity in some brain regions, namely the left superior frontal gyrus, left precuneus, superior parietal lobule, superior portions of the right inferior parietal lobule and the right lingual gyrus (Schweinsburg et al., 2005). These brain regions are involved in tasks such as self awareness (superior frontal gyrus), perception of emotions of the face (superior right inferior parietal cortex) and the detection and interpretation of sensory and visual stimuli, visual spatial imagery and spatial orientation, all tasks that must develop early in the infants life to allow interpretation of the world around them and as such would reach peak activity and be stabilised during childhood. This increase in activity is considered to reflect the maturation of the brain networks subserving these functional tasks.

Tasks designed to measure brain activity during behavioural suppression, such as suppression of context-inappropriate behaviours, show maturation in a similar way. The oculomotor response suppression task caused increased activation of frontal, parietal and thalamic regions with increased age (Luna et al., 2001). Adolescents showed greater activation of the prefrontal cortex than
both adults and children. This result appears to be indicative of the high activity of the prefrontal cortex while connections are formed and stabilised prior to maturity.

1.2.5 Selective Stabilisation of Synapses

At the cellular level, stabilisation of a brain network requires stabilisation of the connections between component neurons and, based on both grey matter changes and functional MRI data, adolescence is the developmental period during which this occurs in the prefrontal cortex. To reach the adult state of maturity, each neuron of the brain must make thousands of functionally significant connections (Drachman, 2005). These connections were thought to be predetermined by the individual’s genetic code; however, the theory of selective stabilisation suggests that the precise connections made between neurons during development cannot possibly be entirely genetically controlled (Changeux and Danchin, 1990). Rather, connections are made in abundance early in development and are removed or strengthened on the basis of their usage (Changeux and Danchin, 1990).

Depolarisation of neurons causes an increase in intracellular Ca²⁺ concentration, which can result in both immediate and long-term dendritic stabilisation. Thus the environment, by way of activity that
exposure to the environment elicits, can influence the synaptic elimination and dendritic pruning by strengthening active synapses (Lohmann and Wong, 2005). Over the course of an individual's lifetime, their synapses will be constantly altered, this is one mechanism that allows us to learn, however the refinement of each brain region occurs during certain developmental windows and it is during adolescence that the greatest amount of stabilisation occurs in the prefrontal cortex. It is important to note that environmental stimulation to establish brain circuitry may include abnormal or ‘non-optimal’ stimulation of the brain and it is now widely thought that drugs of abuse that interact with the specific receptors at the synapse and thus alter synaptic function could have important and long lasting consequences when used during adolescence.

1.2.6 Synapse Changes in the Prefrontal Cortex

Electron microscopic studies of the rhesus monkey have provided evidence that developmental synapse numbers change, as postulated by Changeux and Danchin (1990). A rapid increase in synaptic density occurs in the prefrontal cortex occurring from before birth until two months after birth (Bourgeois and Rakic, 1993) with the synaptic density remaining at a high level up to the age of three years, at which point it began to decline. Studies of hormonal
changes suggest that adolescence in rhesus monkeys occurs between 2 and 5 years (Allen et al., 2014).

Synaptic density in human brains has also been studied, however sample sizes are often very limited due to a limited availability of suitable material. Electron microscopy studies of synaptic density in the prefrontal cortex in humans have provided valuable information about synaptic stabilisation. Huttenlocher et al. (1979) reported a significantly higher, variable, synaptic density in children aged between 6 months and 7 years compared to a stable number of synapses between the ages of 16 and 72. Peak synapse number occurred at age 5, before a steep reduction in synaptic number, which reached a plateau during adolescence. Further research suggested peak density was reached at 15 months in the prefrontal cortex (Huttenlocher, 1997). The later study included several individuals between the ages of 12 and 19, to address a lack of adolescent time points in the 1979 study, and suggested that synaptic density stayed high until synapse elimination began at mid-adolescence (Huttenlocher, 1997).

As human tissue for these types of studies is hard to obtain, the above studies used small sample sizes and usually only one individual representing each age. This, therefore, makes it difficult to
understand normal individual variation in synapse number in humans.

### 1.2.7 Behavioural Maturation in the Adolescent

As described earlier, synapse generation is a genetically controlled developmental event but stabilisation is dependent on synaptic use, which occurs in the animal/human during the execution of behaviour. During the adolescent years the brain matures and formal operational thinking comes to dominate over the concrete operational thinking that is characteristically seen in children. This allows the abstract thought, reasoning, motivation, judgement and planning seen in adults (McAnarney, 2008), which are considered to be a function of the prefrontal cortex. Both functional MRI and synaptic density studies have shown that the prefrontal cortex matures during mid to late adolescence making it one of the last brain regions to develop, which correlates with the developmental time line of these so-called adult behaviours (Baumrind, 1987; McAnarney, 2008). Risk taking, exploration, novelty, sensation seeking, social interaction and play behaviours are more frequent in adolescents than other ages and are an important part of their development (Baumrind, 1987; Hol et al., 1999). These behaviours are seen in most mammals and are thought to be an
evolutionarily conserved aspect of development, as the dangers implicit in some of these behaviours are outweighed by a necessity for the individual to explore and learn as it reaches the age where it can no longer rely on the parental protection it had as a child (Baumrind, 1987; Hol et al., 1999). Risk taking and exploratory behaviours during this period, especially in human mammals (although data in rats supports this idea) such as drug taking and alcohol consumption, may impact on adolescent brain development, as behaviour is the factor determining structural change.

1.3 The Effects of Alcohol on the Developing Adolescent Brain

As mentioned above, explorative behaviours and risk taking are essential behavioural experiences that support normal adolescent brain development but in recent years the use of alcohol and drugs of abuse has become associated with risk-taking and has become a risk taking behaviour itself (Leigh, 1999; Spear, 2000; Crews, et al., 2007). In many societies drugs of abuse are not considered to include alcohol and it is thus difficult to disentangle the role of alcohol from other drugs. Research groups and policy makers have now begun to question whether or not the adolescent brain is differentially
susceptible to the effects of alcohol and other drugs of abuse due to its very plastic nature (Spear, 2000; Odgers et al., 2008). This is a very important consideration as the ability to carry out complex mental tasks is dependent on the prefrontal cortex that develops during adolescence and the dendritic pruning and synaptic stabilisation essential to this process may be altered by the changes in brain activity that occur in the presence of drugs, such as alcohol. This would result in the brain being wired in a state of alcohol or drug function, not in the normal state.

A literature search revealed that the scientific literature was almost devoid of publications investigating the effects of alcohol on the adolescent brain prior to the mid 1990s. This research area has grown considerably in recent years, and the following discussion will cover the main threads of current research in the field of adolescent alcohol exposure.

1.3.1 Alcohol’s Effect on the NMDA and GABA receptors

Glutamate is the most widely used neurotransmitter in the brain and acts via a range of receptors including the N-methyl-D-aspartate (NMDA) receptors that are found at synaptic and extra-synaptic sites of neurons as well as glial cells (Paoletti and Neyton, 2007). NMDA receptors are protein complexes that form a ligand-gated ion channel
from four subunits, which under resting conditions is blocked by Mg$^{2+}$ ions. The specific activity of the receptor is determined by the specific composition of the channel, using the subunits NR1, NR2A-D or NR3A-B. This receptor is important in neural plasticity such as is seen in learning and memory (Castellano et al., 2001; Riedel et al., 2003)

Ethanol acts on NMDA receptors and GABA, receptors as well as a variety of other receptors, ion channels and signalling pathways. Intoxicating concentrations of ethanol inhibit NMDA receptor activity, and electrophysiological studies using hippocampal brain slices have shown that the ethanol molecule is a potent antagonist of NMDA-mediated neurotransmission (Woodward, 2000; Allgaier, 2002; Vengeliene et al., 2008). The ethanol molecule is inhibitory, it decreases flow, in a dose-dependent manner, through ion channels that are mediated by the NMDA ligand-gated receptor (Lovinger et al., 1989) resulting in downstream effects such as the attenuation of long-term potential (LTP), the mechanism that allows long-term changes in neurons’ reaction to depolarisation. Pyapali et al. (1999) have demonstrated the suppression of LTP in vitro as a result of exposure of hippocampal slices from PN30 rats to ethanol at 10mM and 30mM concentrations. This was in contrast to a lack of LTP suppression by alcohol in PN90 rat hippocampal slices. N-methyl-$\gamma$-
aspartate-mediated neurotransmission is important in the consolidation of synaptic connections made during early brain development (Constantine-Paton, 1990). Thus attenuation of NMDA-mediated neurotransmission may decrease the consolidation and stabilisation of synaptic connections resulting in decreased connections and the neural and cognitive impairments associated with exposure to alcohol (Lovingier et al., 1989). It is important to note that NMDA receptor subunit composition changes during development, which may subserve the differential effects of alcohol on the receptor at different developmental stages (Williams et al., 1993). Population excitatory post-synaptic potential amplitudes in the CA1 region of the hippocampus were significantly lower in brain slices taken from juvenile rats compared to adult rats (Swartzwelder et al., 1995). At all concentrations of ethanol, from 10mM to 100mM, there was a significant decrease in amplitude in the juvenile group whereas only the 100mM concentration caused a significant decrease in the adult brain (Swartzwelder et al., 1995) indicating that the juvenile animals were more sensitive to the effects of alcohol on this receptor. The subunit composition of the NMDA receptors can be altered by exposure to alcohol, which will in turn result in changes in the NMDA receptor mediated excitation. Whole cell voltage clamp recording in the bed nucleus of the stria terminalis, in brain slices
from C57B/6J mice, found that the intermittent, but not continuous, exposure to ethanol vapour resulted in an increase in excitatory post-synaptic current due to an increase in NR2B-subunits within the NMDA receptors (Kash et al., 2009).

It has been well established that chronic exposure to alcohol results in an increase in number and/or function of NMDA receptors (Samson and Harris, 1992; Fadda and Rossetti, 1998; Kumari and Ticku, 2000, Allgaier, 2002). This is considered to be an adaptive response to prolonged attenuation of receptor activity during the continued presence of the inhibitory ethanol molecule. Most studies have used a chronic exposure paradigm and it is unclear how many continuous days of exposure are required to elicit an upregulation of NMDA receptors (Carpenter-Hyland et al., 2004). It has also been found that significant neuronal death occurs in cultured hippocampal neurons after two days of chronic exposure to 100mM ethanol but not one day of exposure (Smothers et al., 1997).

This finding explains the seemingly conflicting observation of alcohol having an inhibitory effect on NMDA receptor activity but also resulting in an increase in extracellular levels of the neurotransmitter glutamate during withdrawal from chronic alcohol exposure. Excessive extracellular glutamate can lead to an increase in Ca\(^{2+}\) influx into the cell, which can cause excitotoxicity (Chandler et
The Ca\(^{2+}\) causes the activation of proteases, lipases, endonucleases or nitric oxide synthases; oxidative stress; and potentially irreversible damage to the mitochondria (Yang et al., 2014; Oster et al., 2014) that ultimately results in neurodegeneration (Manev et al., 1989; Hu, 2012).

Although much of this work has been carried out in cell culture preparations with neurons extracted from neonatal rat brains or in organotypic slices, it is suggestive that the developing adolescent brain may be at risk due to excessive alcohol exposure. Alcohol exposure of the adolescent brain may result in depression of ongoing activity, and thus, reduced stabilisation of normal cortical circuitry. However it may also result in an upregulation of NMDA receptors and due to the role of NMDA receptors in synaptic stabilisation, circuits involved in alcohol consumption and the reward systems associated with consumption may be stabilised. Conversely rather than affecting the connections between the neurons, the upregulation of NMDA receptors, during withdrawal from intoxicating levels of alcohol consumption, may result in excitotoxic cell death of these neurons.

All of these scenarios have implications for the normal development of the adolescent prefrontal cortex and show that the outcomes may be variable depending on the age at which exposure
to alcohol occurs and on the amount, frequency and duration of alcohol exposure. It is also possible that both aberrant stabilisation plus excitotoxic cell death may occur as adolescent drinking favours a pattern of binge drinking that is characterised by multiple rounds of intoxication with subsequent withdrawal.

Ethanol also interacts with the GABA<sub>A</sub> receptors resulting in a decrease in inhibitory input during periods of intoxication. As seen in the NMDA receptor, chronic intermittent alcohol intoxication models have demonstrated a change in GABA<sub>A</sub> receptor function and subunit composition in the adult rat hypothalamus (Cagetti et al., 2003) as well as increased anxiety; tolerance to GABAergic sedative drugs; and measurable reductions in allopregnanolone, a neurosteroid that potently enhances GABA<sub>A</sub> receptors in the hippocampus (Cagetti et al., 2004). GABA<sub>A</sub> receptor subunit expression patterns change during adolescence (Yu et al., 2006) and alcohol exposure during adolescence causes changes in allopregnanolone levels that last into adulthood (Silvers et al., 2006).

1.3.2 Adolescent Versus Adult Responses to Alcohol Sedation

Adolescents are less susceptible to many of the negative effects of alcohol, despite increased in vitro susceptibility to the attenuation of
NMDA-mediated neurotransmission. Recovery of the righting reflex (return of the rat to its normal upright body position after being placed on its dorsal surface) has been used to assess the sedative effects of alcohol on differently aged rats. This was significantly decreased in adolescent rats, after an ethanol dose of either 4 or 5g/kg, compared to adult animals, despite a higher serum alcohol concentration in PN30 rats at the time of righting (Little et al., 1996; Swartzwelder, 1998). In the above study, adolescence was modelled with a PN30 animal. It is generally accepted in the scientific literature that adolescence in the rat begins around PN25-30 and lasts until around PN45-50 as these ages model the structural and functional brain changes observed in human adolescents (Crews et al., 2000; Spear, 2015). Another study examined loss of righting reflex duration in rats of ages PN16, 26, 36, 46, 56 and 96 after a single hypnotic dose of alcohol. Duration was shortest in PN26 animals with durations increasing with increasing age (Silveri and Spear, 1998). After a dose of 2.5g/kg, adult but not adolescent rats suffered locomotor dysfunction and in response to all doses, the peak serum alcohol concentration was achieved earlier in adolescent rats (Little et al., 1996). This difference has the effect of allowing adolescents to continue drinking alcohol after an adult animal would have succumbed to its hypnotic effects. Theoretically, this means that
there is more danger of so-called binge drinking as the adolescent could continue to imbibe to higher blood alcohol levels than recorded for adults.

Changes in motor coordination during alcohol exposure also illustrate differences in the susceptibility of the two age groups to alcohol. There were no baseline differences in performance on the task, which was a test of a rat’s coordination as it attempts to not slide down an increasing incline, but an intraperitoneal injection of both 2.0g/kg and 3.0g/kg ethanol had a greater effect on the performance of adult than adolescent animals. Furthermore, at almost all time points from 15 to 180 minutes, adolescent rats were performing significantly closer to their baseline performance than adult rats (White et al., 2002). Male mice, aged PN34-35, were also more resistant to an alcohol-induced reduction in locomotor activity than PN70 adult male mice (Lopez et al., 2003).

Gavage delivery of ethanol, 4g/kg delivered twice daily 12 hours apart for either three or seven days, was used to compare the tolerance to hypothermia caused by alcohol at different developmental stages (Swartzwelder et al., 1998). Thirty-six hours after the final gavage delivery, animals were challenged with a single 5.0g/kg intraperitoneal dose and there was no tolerance to the hypothermia caused by the acute alcohol in either age, however
some tolerance was evident in adolescent animals between the third and seventh day of alcohol pre-treatment.

These results suggest that the adolescent brain is less sensitive or recovers more quickly from the sedating effects of alcohol, suggesting that these animals may be able to continue consuming alcohol past the point at which an adult animal would have succumbed to this sedation.

1.3.3 Age Related Effects on Brain Function

Adolescents and adult rats also show differences in learning ability in response to alcohol. Performance on memory tasks shows a differential response to alcohol exposure between adolescent and adult animals given 1.0g/kg, 2.0g/kg or no alcohol. The 1.0g/kg dose of ethanol was enough to increase latencies and path lengths to the hidden platform during training trials of the Morris water maze reference memory task in adolescent animals with adults not affected at either dose (Markweise et al., 1998). The subsequent probe trial found that the adolescent rats, treated with the 2.0g/kg ethanol dose spent significantly less time in the target quadrant than the control treated littermates, while adult animals given either alcohol dose showed no performance deficit at either dose (Markweise et al., 1998).
1.3.4 Adolescent Alcohol Exposure and Brain Damage

Brain function is altered by exposure to alcohol and in this study we are investigating whether exposure to alcohol results in long-term functional changes that may be related to structural brain changes.

Brain activation patterns, as assessed with fMRI, were used to compare spatial working memory performance in human adolescents aged 14-17 years with alcohol use disorders (AUDs), at least 72 hours since intoxication with control adolescents matched for age, sex and demographic background. The activation pattern in frontal brain regions during the spatial working memory task was significantly different in AUD adolescents (Caldwell et al., 2005). There was a significant interaction between AUD diagnosis and sex in superior frontal, superior temporal, cingulate and fusiform regions with female AUD subjects showing a greater departure from control patterns of activation than males with AUDs (Caldwell et al., 2005). Heavy drinking for 1-2 years did not affect working memory performance despite differential brain activation patterns but a longer term of heavy drinking resulted in decreased performance as well as differential brain activation patterns. The authors suggest that this is clear evidence of reorganisation of brain circuitry, in response to neuronal disruption caused by alcohol. Although the
study did preclude individuals with a history of neurological disorders, traumatic head injuries and poly-drug abuse, it was not able to preclude individuals with conduct disorders, as these were so often comorbid with AUDs. Therefore it is difficult in studies, where AUD diagnosis has been used to determine inclusion in the adolescent alcohol-exposed group, whether the alcohol is the causative factor or there was a pre-existing brain condition that determined whether or not the individual became an early alcohol user. However, if the authors were correct in their suggestion that alcohol caused a reorganisation of brain circuitry, there is potential for subtle but long-lasting effects in the drinking population at large, and further study is necessary.

This issue has been further elucidated in a prospective study that followed a cohort of infants from birth for 30 years, finding that early exposure to alcohol and illicit drugs (mainly cannabis) before the age of 15 almost doubled the risk of becoming an adult with substance dependence or other negative life outcomes (Odgers et al., 2008). This study, having selected a group of individuals with no prior history of conduct disorder, suggesting no propensity to drug use, strongly suggests that alcohol exposure during early adolescence alters brain structure. A range of animal models have been used in studies, that pre- and post-date the Odgers et al. (2008) finding, to
further investigate whether adolescent alcohol exposure results in long-term changes in brain structure and function. These animal models primarily use rats and have contributed most of what is now known about adolescent binge drinking.

One widely used rat model delivers 9-10 grams of ethanol per kilogram per day, for four consecutive days, via gavage, administering the alcohol as four doses, eight hours apart with dose titrated to maintain moderate inebriation. This model has found that the adolescent brain exhibits neurodegeneration, visualised with the amino cupric silver stain, that is more severe and more widespread than that seen in adult animals exposed to the same regimen (Crews et al., 2000). Neurodegeneration was evident in the frontal cortical olfactory regions as well as anterior piriform and perirhinal cortices (Crews et al., 2000). A single 5.0g/kg dose of ethanol resulted in almost no staining, no degeneration, suggesting that prolonged exposure to alcohol may be required to elicit cell death. Pascual et al. (2007) used an alternative rat model where ethanol, 3.0g/kg/day was delivered via a single intraperitoneal injection, on two consecutive days, followed by two alcohol free days throughout adolescence, from PN25 to PN38. This model attempted to mimic more accurately the type of alcohol exposure seen in human adolescents who commonly binge drink on weekends. They found a
significant increase in apoptosis in the neocortex, hippocampus and cerebellum 24 hours after the cessation of ethanol exposure, indicating that alcohol damages the adolescent brain. These two important studies indicate that alcohol exposure during adolescence appears to selectively damage frontal regions of the brain that develop later in adolescence.

1.3.5 Glial and Inflammatory Response to Alcohol

In general, an insult to the brain causes inflammation and gliosis, a reactive change in glial cells, at the site of the injury. When alcohol is administered during adolescence or adulthood, there is activation of glial cells along with induction of inflammatory mediators cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Pascual et al., 2007, Alfonso-Loeches et al., 2013). This parallels apoptotic cell death in the neocortex, hippocampus and cerebellum (Pacual et al., 2007). However, when 4mg/kg of indomethacin, a non-steroidal anti-inflammatory drug and inhibitor of COX-2, was administered immediately before alcohol dosing, there was no significant induction of COX-2 or iNOS in the neocortex or hippocampus (Pascual et al., 2007). There was also no significant increase in apoptotic cell death in the neocortex, hippocampus or cerebellum and behavioural deficits that were present in animals
that received only alcohol were ameliorated in animals that were also given indomethacin (Pascual et al., 2007). Activation of the inflammatory cascade, the COX-2 pathway, plays a key role in the induction of apoptotic cell death in the adolescent brain following ethanol exposure. Gliosis appears to be a key factor in alcohol-induced brain damage and has been shown throughout the brain where cell death, imaged using FluoroJade B to stain for neurodegeneration, is present, in adult male rats that were exposed to a four-day binge of alcohol (Kelso et al., 2011). In adults, ethanol acts as an agonist of Toll-like receptor 4 (TLR-4) (Blanco et al., 2005, Fernandez-Lizarbe et al., 2009) on glial cells, causing activation of the TLR-4, production of cytokines and inflammatory mediators such as COX-2 and iNOS, neuroinflammation, and neurodegeneration (Alfonso-Loeches et al., 2013). In TLR-4 knockout mice, there is no significant difference in activation of iNOS, COX-2 or caspase-3 when treated with alcohol (Alfonso-Loeches et al., 2013). A recent study demonstrates only partial activation of microglia, presenting [H]-PK-11195 and OX-42 and not OX-6 and ED-1 immune proteins in response to a four-day alcohol binge that causes neurodegeneration (Marshall et al., 2013). Also present were anti-inflammatory cytokines and growth factors but not pro-inflammatory cytokines. Therefore in this instance, only partial
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Microglial activation occurred although it occurred in the presence of neurodegeneration. In other studies, chronic alcohol exposure has caused gliosis in alcohol-treated adult rats (Alfonso-Loeches et al., 2013) but this also occurs in response to only 24-hours of elevated BEC (Hayes et al., 2013). Interestingly, in adult animals, while chronic alcohol treatment caused increased levels of inflammatory mediators, cytokines, gliosis processes, caspase-3 and neurodegeneration, levels of the above were significantly higher in female compared to male animals (Alfonso-Loeches et al, 2013) suggesting that female adults are more susceptible to ethanol’s effect on this inflammatory cascade. It is still not clear what the exact role of gliosis and inflammation is in alcohol-induced neurodegeneration and it is likely that the mechanisms are different in the adolescent compared to the adult.

1.3.6 Alcohol Disruption of Neurogenesis

Neurogenesis, the production of new neurons in the brain is known to continue through adulthood, and occurs in the dentate gyrus of the hippocampus (Altman, 1963; Kaplan and Hinds, 1977; Eriksson et al., 1998), the sub ventricular zone (Sanai et al., 2004) and the olfactory bulb (Kaplan and Hinds, 1977; Bedard and Parent, 2004). The importance of the functional significance of adult born neurons
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is of considerable interest and generates considerable debate (Rakic, 1974; Rakic, 1985; Kornack and Rakic, 2001; Bhardwaj et al., 2006)

A study by Morris et al. (2010) suggests that the reduction of neurons in the hippocampus that is seen in human adolescents with AUD is likely to be due to decreased neurogenesis in this region. Continuous alcohol treatment from PN35 to PN39 resulted in a decrease in neurogenesis, determined by a decrease of 33 and 28% of doublecortin expression, at 0 and 2 days after the final dose of ethanol respectively (Morris et al., 2010). BrdU labelling of dividing cells revealed a 22% decrease in alcohol-exposed brains. Moreover, 28 days after the final dose, there were 53% fewer BrdU labelled cells in the dentate gyrus of in the alcohol exposed animals (Morris et al., 2010). The difference between the BrdU labelling at 0 and 28 days after alcohol treatment suggests that both cell proliferation and cell survival were affected by alcohol (Morris et al., 2010). Other studies have supported the finding that alcohol alters normal cell division and migration during adolescence reducing adult neuron and glia numbers (Koss et al., 2012) and actively dividing cell types (Taffe et al., 2010), as well as increasing proliferation during withdrawal of both neurons (McClain et al., 2014) and glia (Nixon et al., 2008). Some new cells created during adolescence in the dentate gyrus differentiate into glia, however the Morris et al. (2010) study showed
no changes in glial outcomes in response to alcohol. Neurogenesis disruption in response to alcohol is very likely dependent on the length of exposure and the alcohol dose as a two-day binge model failed to replicate the above findings (Hayes et al., 2013).

Clearly, there are many ways in which alcohol can impact the adolescent brain, making it an area worthy of investigation, particularly using a model that is representative of the drinking pattern and delivery mode seen in adolescents.

1.4 Adolescent Binge Drinking in New Zealand

Binge drinking is a common pattern of drinking and is defined as the consumption of seven or more drinks in one drinking session (Fryer, et al., 2011). Binge drinking results in high blood ethanol concentrations (BECs) and is potentially more damaging to the brain than chronic drinking (Becker, 1997). Currently, binge drinking is common in young people who are 'drinking to get drunk' and therefore using alcohol as a drug of abuse (Kypri and Langley, 2003).

Alarming statistics are emerging pertaining to heavy drinking in secondary and tertiary students. A report released by the Alcohol Advisory Council of New Zealand (de Bonnaire et al., 2004), found that 53% of young people aged between 12 and 17 years old classify themselves as 'current drinkers,' with 11% drinking weekly and 37%
drinking more than 5 drinks in their most recent drinking session. As drink size is commonly underestimated compared to the standard drink containing 10g of ethanol, such drinking could potentially be classified as binge drinking (Caswell et al., 1994). Furthermore, 61% of the young people surveyed reported concern about the long-term effects that drinking may have on their internal organs such as the liver and the brain (de Bonnaire et al., 2004). A longitudinal study from Christchurch studied individuals from birth for 16 years to investigate the prevalence of adolescent alcohol abuse, finding that between 7% and 10% of the sample reported drinking patterns that were indicative of ‘frequent, heavy or problem-related drinking’ (Fergusson et al., 1995)

A web-based survey of tertiary students at 8 New Zealand institutions with an average age of 20.2 years revealed that 22.8% had had an episode of binge drinking in the previous week, 14.1% had had two or more episodes and 68% of those surveyed scored 4 or above on the AUDIT-C scale, indicating hazardous drinking (Kypri et al., 2009). Tertiary students who reported binge drinking greater than monthly during high school were at 6 and 20 times the risk of binge drinking once or greater than two times respectively over the seven days prior to the survey compared to students that did not drink during high school (Kypri et al., 2009). In New Zealand
a revision to the Sale of Liquor Act in 1999 meant that the legal purchase age was lowered from 20 to 18 years in the face of health research evidence that drinking in this age group is a serious concern and worthy of government action (Mason et al., 1998).

Sociological research, notably that of Skog (Skog, 1980, 1985) suggests that an individual’s drinking habits tend to reflect the drinking habits of their peers. When large numbers of young people are brought together for tertiary study this may introduce moderate drinkers to more binge-like patterns of drinking. Students were randomly chosen to take part in a web-based survey of their alcohol use and were then asked to estimate the incidence of heavy drinking in: young people in New Zealand of the same age and gender; local university students of the same age and gender; and the closest friends of each participant (Kypri and Langley, 2003). Eighty percent of women and 73% of men overestimated the incidence of heavy drinking among student peers. Interestingly, overestimation of peer drinking was strongly associated with the individuals own frequency of heavy drinking and only 9% of the participants believed that they drank more than other students. Diaries completed by participants revealed that incidence of episodic weekend heavy drinking was 45% for men and 36% for women (Kypri and Langley, 2003).
A study by Kypri (Kypri et al., 2002) focussed on the halls of residence in Dunedin, New Zealand. Alcohol use was high, with around 60% of both male and female respondents exceeding the current ALAC guidelines of 6 drinks for males and 4 for females per occasion (Kypri et al., 2002). Male respondents reported a mean of 2.4 drinking episodes per week compared to the female mean of 2.0 episodes per week. Male respondents also reported consuming a mean of 8.5 standard drinks per occasion compared to a mean of 5.5 standard drinks per occasion in women. Extreme levels of drinking were defined in the study as drinking 16 or more standard drinks in an occasion in the preceding 4 weeks and this was reported in 33.6% of males and 7.3% of females. AUDIT scores indicated that the majority (62.4% of men and 47.6% of women) of respondents scored over 8, indicating hazardous or harmful drinking. Of particular interest, the frequency of blackouts, having no memory of at least parts of the drinking occasion, among participants was 36.9% of males and 33.2% of females in the three months prior to the survey.

It is clear that adolescent humans commonly engage in binge drinking, and human and animal data indicates that this can result in acute damage and negative life outcomes. Data obtained from animal model studies can be used to inform about the likely outcome in the human population as long as the human situation is faithfully
modelled. When major discrepancies exist in the most influential adolescent alcohol exposure models relating to pattern and method of alcohol delivery is can be misleading to extrapolate their findings to the human population. For this reason additional, more relevant, animal model data is required.

This thesis investigated the effects of exposure to ethanol during adolescence on both the structure and function of the brain using a rat model.
1.5 Experimental Aims and Hypotheses

1.5.1 Overall Aim

The overall aim of this study was to investigate the effects of binge exposure to alcohol during adolescence using a rat model.

A key aspect of this study was that ethanol was delivered via intragastric intubation using a periodic binge regimen throughout adolescence. The time points of interest during adolescence covered acute and long-term exposure at each of days, PN28 and every fourth day until PN48, with cumulative exposure until one group of animals were exposed on each of the days. A high dose (9.0g/kg/day) was used, as the primary objective was to model youth that are considered heavy binge drinkers. Acute cell death, glial cell response and long-term behavioural performance, were all investigated in a series of experiments.

1.5.2 Hypotheses

The hypotheses for this study are specific for each experimental objective so are listed as such below:
1.5.2.1 Experiment One

Overall aim: Develop the specific parameters of the periodic binge rat model.

1. Moderate to severe intoxication with BECs between 300 and 500g/dL will be achieved with an ethanol dose of 9.0g/kg/day.
2. Animals in the alcohol-treated group will show both behavioural and metabolic tolerance to alcohol.

1.5.2.2 Experiment Two

Overall aim: Investigate the effects of repeated binge alcohol exposure on neuron and astrocyte appearance and number.

1. Ethanol exposure will result in a significant increase in apoptotic cell death after a single binge exposure (PN29) and at the cessation of multiple exposures (PN49).
2. Ethanol exposure will result in reactive gliosis and this will be maximal after repeated binge exposure as seen in PN49 animals.
3. The total number of astrocytes, determined using unbiased stereological methods, will be greater in animals exposed to repeated binge alcohol exposure. There will also be significant hypertrophy of astrocytic processes in alcohol-exposed animals.
1.5.2.3 Experiment Three

Overall aim: Investigate the long-term effect of repeated binge alcohol exposure on a number of behavioural measures.

1. Alcohol exposed animals will show greater anxiety than control animals.
2. Alcohol exposed animals will show working-memory/attention deficits compared to control-treated animals.
3. Alcohol-treated animals will show deficits in hippocampal based spatial learning.
2 Experiment One
Chapter Two: Experiment One

2.1 Materials and Methods

2.1.1 Animals and Treatments

Time-mated, where the date of vaginal plug was gestational day zero (G0), female Long-Evans rats were obtained from the Hercus Taieri Animal Resource Unit, University of Otago. Dams were transferred to the Hercus building, HTRU, University of Otago on G17. Pups were born at around G22, therefore known as postnatal day zero (PN0), and dams and pups were maintained on a 12-hour light/dark cycle with access to food and water ad libitum. Between PN2 and PN6 pups were sexed and the litter culled to 12 pups, ideally six male and six female. Pups of both sexes were used in the study. At this time pups were also paw marked or ear marked (see Appendix A). Pups were weaned on PN25 and housed in within litter, same sex groups under standard conditions. On PN27, the evening prior to the first day of alcohol treatment, cages were moved from the Hercus building, to a temperature and humidity controlled housing chamber (for litters five to eleven) in the Lindo Ferguson Building, University of Otago where dosing would take place.

Litters One to Four were treated in the laboratory where they were kept for the day of treatment and the night following treatment before being moved back to the Hercus building, University of
Otago. These litters were transferred back to the Lindo Ferguson Building on the morning of each experimental treatment. Transfer of animals was achieved in the evenings and early mornings using approved rodent cage bags and trolleys to reduce exposure to external cues. The light cycle could not be controlled during the alcohol treatment period but all treatment groups were exposed to the same lighting conditions and lights on/off times in the laboratory where testing took place varied by a maximum of two hours. Animals to be included in the adult behavioural testing experiment (see Chapter Four) were returned, after the final day of experimental treatment, to the Hercus building and were kept under the conditions of this facility (as described above) for a period of between 284 and 311 days. One week prior to the commencement of behavioural testing, on PN333-360, the animals were moved to the Behavioural Phenotyping Unit (BPU), University of Otago, for housing and experimentation. The BPU is a purpose-built area for the housing and phenotyping of animals for behavioural experiments. Rats were housed in Plexiglas cages (AirLaw Pty Ltd., Australia) attached to a ventilation system designed to control temperature and humidity as well as eliminate olfactory cues from other cages. The rats were kept on a 12-hour light/dark cycle with lights on at 7am. Rats had access to food and water ad libitum. The
University of Otago Ethics Committee gave ethical approval for this project AEC20/10.

Three treatment groups (one alcohol and two control groups) were used for this study. Animals in the alcohol group (E) were given a dose of 9g/kg/day as four gavage deliveries of 20% ethanol w/v solution 15 minutes apart. Intubated (IC) and non-intubated (NIC) control groups were used to control for a possible stress response to the gavage and tail clip events that could have an effect on brain histology and behaviour. When these two control groups were found to not be statistically different, the groups were combined to increase statistical power. Due to experimental design this also allowed sex differences to be investigated.

In order to minimise any possible stress, animals were handled every second day from PN20-27 in order to habituate the animals to the stress of handling during experimental treatment. The early handling made the animals amenable to the gavage hold but the gavage delivery of fluid or sham gavage still appeared to be aversive. We could not eliminate this as the volume of the gavage caused stomach distension (as concluded by the University of Otago veterinarian), which may have caused discomfort or pain. Animals were used for either behavioural testing (see Chapter Four) or analysis of tissue structure (see Chapter Three).
2.1.2 Experimental Treatment

2.1.2.1 Gavage Procedure

The gavage delivery began at 8:30AM ±90 minutes for all experiments. Intubation was performed using a modified stainless steel gavage needle. The bulb was reduced in diameter and highly polished (see Figure 2.1) for smooth insertion through the cardiac sphincter into the stomach.

![Modified stainless steel gavage needle used (below) compared with standard needle (above). Note the bulb has been reduced in size and polished.](image)

Figure 2.1 Modified stainless steel gavage needle used (below) compared with standard needle (above). Note the bulb has been reduced in size and polished.

Two sizes of gavage needle were used so that the younger, smaller animals could be effectively intubated without discomfort. During the gavage procedure, animals were restrained using a towel. With the towel covering the experimenter's hand, the rat was put into the palm of the hand and the towel was wrapped upwards around the
animal. It was wrapped tightly enough to immobilise the forepaws and hold the head still while still allowing the animal to breathe normally. The animal was held vertically in the towel and the gavage needle was inserted into the side of the mouth and passed over the back of the tongue. With the animal swallowing, the gavage needle was allowed to slide down the oesophagus under its own weight into the stomach. Correct insertion was determined by gently pulling back on the syringe, resistance indicating that the bulb was in the stomach. The time of each gavage was recorded along with any notes concerning reflux or behaviour of the animal.

Over the course of experiments, a total of 11 litters were intubated. Records pertaining to deceased animals were recorded (see Appendix B).

2.1.2.2 Alcohol Treatment Regimen

The intubation procedure began on PN28 and rats were given alcohol every fourth day until PN48. Therefore alcohol delivery days were PN28, 32, 36, 40, 44, and 48. In the three days between alcohol treatments, rats were carefully monitored and animals that were slow to gain weight were hand-fed Kellogg’s Froot Loops™ until normal weight gain was achieved.
During the evening prior to the first alcohol treatment day the rats were weighed using electronic scales. The weights were recorded alongside the ear mark/paw mark number for each animal throughout the experiment. The weights were transferred to the daily dose calculation sheet (see Appendix C) which was used to weight match the animals across sexes. Animals in the alcohol gavage group were given 9g ethanol (diluted in distilled water) per kilogram body weight delivered as four, equal volume gavages, 15 minutes apart spanning 45 minutes. Animals in the alcohol group were then given tail mark identification for rapid identification and retrieval on alcohol days. It was noted that rats tended to disregard their tail when grooming so markings would last at least four days and were darkened each time the animals were weighed for alcohol dosing. Tails were marked in red or blue for alcohol gavage group and green for control gavage group. Non-intubated controls did not need to be marked, as they were not removed from the cage. Marking consisted of a series of parallel lines near the base of the tail.

2.1.2.3 Control Treatment Regimen

Intubated controls had a clean, empty gavage needle inserted into the stomach and held in place for 10 seconds. Intubated controls were exposed to the same handling-induced stressors as the ethanol
treated rats. Non-intubated controls were not disturbed during the experiments and remained in the cage. All animals were housed with at least one member of each treatment group per cage.

2.1.3 Intoxication State

Prior to each blood sampling, the duration to complete the righting reflex was measured as a behavioural measure of intoxication. The rat was held (lying on its back) on a paper towel, and then released and the time taken for it to roll over with all four paws on the ground was recorded. If the animal had not righted itself within 60 seconds this was recorded as 60+. A distinction was made within the ‘60+’ group between animals that attempted to right themselves and those that did not as there needed to be a clear distinction between animals that were comatose and animals that were not. These times were later converted into a 6-point scale, which was adapted from Nixon et al. (2008) (see Table 2.1) to compare results with this active research group.
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2.1.4 Blood Ethanol Concentration Analysis

Approximately one millimetre was cut from the tip of the tail using a razor blade. The animal was supported in the hand while 20μL of blood was massaged out of the tail and into a heparinised capillary tube. The blood was transferred into a vial containing 180 μL of internal standard for ethanol content analysis (see Appendix D). Blood samples were taken from all animals (except NICs) using the tail clip method at four hours post the initial intubation. For some animals, blood was taken at 10 hours post the initial intubation.

<table>
<thead>
<tr>
<th>Adapted Scale</th>
<th>Instant righting</th>
<th>1-4s</th>
<th>5-9s</th>
<th>10-59s</th>
<th>60+s attempted</th>
<th>60+s not-attempted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crews Nixon Scale</td>
<td>Normal Rat</td>
<td>Hypoactive</td>
<td>Ataxia</td>
<td>Dragging abdomen</td>
<td>Loss of righting reflex</td>
<td>Loss of eye blink reflex</td>
</tr>
</tbody>
</table>

Table 2.1 Adapted 6-point scale. Points of the scale are shown along the top of the chart, the righting reflex durations relating to each point are matched below, as are the behavioural notes observed by Nixon et al. (2008).
to provide data on the rate of alcohol clearance; thus all animals had a maximum of two blood samples per treatment day. Samples were analysed using gas chromatography by the Institute of Environmental Science and Research (ESR), Upper Hutt. The BEC was determined for each time point by comparison with a set of ethanol standards.

2.2 Results

2.2.1 Animals

2.2.1.1 Body Weight During Treatment Period

Body weights across the treatment period for males and females are shown in Figures 2.2 and 2.3 respectively. There is a significant effect of age \([F(5,282)=169.9, \ p<0.0001\) and \(F(5,207)=129.9, \ p<0.0001\)] on males and females weight, respectively, and there is also a significant effect of treatment on the male rats \([F(1,282)=6.889, \ p=0.0091]\) but not on the female rats \([p=0.4994]\). Bonferroni post-testing reveals no significant weight differences between alcohol and control animals at any dosing age \((p>0.05)\). There was also no significant effect of interaction.
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Figure 2.2  Mean & SEM of male rat body weight across 20-day treatment period

Figure 2.3  Mean & SEM of female rat body weight across 20-day treatment period
2.2.2 Metabolic and Behavioural Tolerance

2.2.2.1 Blood Ethanol Concentration

Figure 2.4, below, shows that the mean blood ethanol concentration (BEC) at peak (4-hours) and at 10-hours after the initial alcohol exposure decreased over the period of exposure.

![Figure 2.4: Mean and SEM of BEC at 4 and 10-hours](image)

Two-way ANOVA revealed that the effect of day (i.e. dosing day) was significant \([F(5,189)=8.953, \ p<0.0001]\) and time (i.e. 4 or 10-hour BEC) was also significant \([F(1,189)=47.64, \ p<0.0001]\) but there was no
significant interaction between time and day \[F(5,189)=0.7077, p=6.183\]. To look at the specific effect of day on the peak BEC achieved, one-way ANOVA was used and revealed a significant effect of day \[F(5,130)=4.806, p=0.0005\]. Tukey’s post-hoc testing revealed that the peak BEC was significantly higher on day 1 of ethanol treatment than on days 3, 4, 5 and 6 \[p<0.05; p<0.01; p<0.05; p<0.05\] (respectively). The BEC at 10 hours after the initial ethanol dose was also analysed using one-way ANOVA and there was a significant effect of day \[F(5,59)=3.652, p=0.0060\]. Tukey’s post-test revealed a significant difference between weight on day one versus day four \[p<0.05\] but there were no other significant differences between days.

2.2.2.2 Ethanol Clearance Rate

The clearance rate of ethanol (see Figure 2.5) was determined for the time period of four to ten hours, for each animal, for days one to four only as there were insufficient data points to include days five and six. Clearance was calculated from the difference in BEC at the 4 and 10 hour time points divided by time. There was no significant effect of day on clearance rate \[p=0.7945\].
2.2.2.3 Behavioural Tolerance

There was a significant effect of day \([F(5,189)=4.331, \ p=0.0009]\) and time \([F(1,189)=17.67, \ p<0.0001]\) on Behavioural Intoxication Scale (BIS) scores as shown in Figure 2.6. Tukey’s multiple comparison post-test revealed a significant difference between 4-hour BIS on day one and day three \([p<0.01]\) and between 4-hour BIS on day one and day four \([p<0.05]\). The proportion of the group at each point on the BIS scale is shown in Figure 2.7.
Figure 2.6  Mean & SEM of BIS at four and ten hours

Figure 2.7  The proportion of animals classified under each point of the BIS scale on day one (x/31) (A) compared to day six (x/14) (B)
2.3 Discussion

2.3.1 Animals

A significantly reduced body weight was observed in male ethanol-treated animals during the treatment period. The alcohol-treated animals likely reduced their food intake while they were intoxicated and therefore lost weight on treatment days. There was no significant difference between the body weights of female alcohol-treated and control animals. It is unknown why alcohol affected the weights, compared to controls, in male but not female animals. The results suggest that these animals were adequately cared for as their weight returned to normal, evidenced by the lack of significant difference between alcohol- and control-treated male or female animals on any single day.

Some animals died during the experiment. This was approximately 23% of the animals and was higher than expected but repeated intubation of small adolescent rats is a difficult process. Details of these animals are found in Appendix B. These deaths were primarily the result of misintubation, which can occur when relatively large volumes of liquid are intubated into a small animal and there is reflux of solution during the gavage process. The solution can then travel into the lungs. Five deaths were due to
extreme comatose reaction and these animals were euthanized for ethical reasons.

2.3.2 Metabolic and Behavioural Tolerance

The hypothesis that rat behaviour, as seen in the intoxication score, will be affected less by alcohol after multiple binges was supported, as BIS on days three and four were significantly lower than scores on day one. However, BIS on the fourth and fifth day were not significantly different and the data suggests a reversal of this earlier tolerance. We can only speculate on the reason for this reversal, as the scores show a sharp linear decrease from days one to three followed by a linear increase from days three to six (see Figure 2.6). The most likely explanation for this phenomenon is that this is caused by a developmental change, as age has been shown to influence the hypnotic effects of alcohol (Little et al., 1996; Swartzwelder et al., 1998). Furthermore, in the Little et al. (1996) study PN30 animals were more tolerant to the hypnotic effects of alcohol, as evidenced by loss of righting reflex (LORR), than both PN20 animals and adult animals. This supports the findings of the current study. It is also possible that this was a behavioural adaptation to an intoxicated animal being repeatedly placed on its back and the animal may move slower as a result of repeated testing.
It is unlikely to be related to different pharmacokinetics of increasing dose volume because there is no matching pattern in the BEC data, which shows tolerance followed by a plateau (see Figure 2.4). In the above studies, the BEC when animals awoke was higher in the young than in older animals indicating that this is a tolerance to the sedative effects of alcohol rather than an increased clearance of alcohol. This decreased susceptibility may be related to the ongoing development of GABA receptors that may be composed of subunit combinations that are less affected by alcohol (Laurie et al., 1992; Moy et al., 1998). Alternatively, it has been suggested that the developing brain has a greater propensity to adapt to the depressive effects of ethanol, switching to contain GABA, receptors composed of less sensitive subunits. This effect has been shown to occur in the adult hippocampus and cortex following chronic alcohol exposure (Devaud et al., 1997; Matthews et al., 1998).

The decrease in BEC at four hours suggests that metabolic tolerance had also developed. However as there was no change in the rate of metabolism of ethanol between four and ten hours, it is likely that the reduction of BEC at four hours across the treatment period must have been the result of a more complex interaction of factors that are known to influence BEC. Tolerance to ethanol can be acute, where the tolerance is to a dose within 24 hours of a previous
dose, or chronic, a diminished response to ethanol over repeated administration over time (Kalant, 1996). Chronic tolerance is investigated in the present study. As the rate of alcohol clearance between four and ten hours does not increase, up-regulation of alcohol dehydrogenase in the liver is unlikely. Increased first pass metabolism (FPM), the metabolism that occurs in the stomach prior to alcohol transport to the liver, could explain these results. The rate of absorption of alcohol from the stomach may have also changed to account for the reduction in four-hour BEC across the treatment period (Lim et al., 1993; Lieber et al., 1994). First pass metabolism is affected by a number of factors such as the number of alcohol deliveries and the influence of food on gastric emptying, however the extent of FPM in rats is controversial (Nau, 1986; Levitt et al., 1997). The amount of food in the stomach has been shown to affect the rate of ethanol absorption and thus BEC profile (Lim et al., 1993; Oneta et al., 1998). The animals in the present study were not fasted, despite evidence that this would give more consistent results, as fasting before alcohol consumption is not common in human adolescents.
2.3.3 Development of Tolerance in Alcohol-Treated Animals

Studies that have administered ethanol via i.p. injection fail to reach a consensus on production of metabolic tolerance in adolescence (Silvers et al., 2003; Silveri and Spear, 2001; Varlinskaya and Spear, 2007) and this appears to be due to the range of exposure paradigms. Varlinskaya and Spear (2007) found that adolescent rats did not develop metabolic tolerance, when exposed to conditions that resulted in tolerance in adults, despite developing behavioural tolerance. Other studies have shown that chronic alcohol exposure results in the development of metabolic tolerance (Silvers et al., 2003). Metabolic tolerance is dependent on the frequency of doses and the size of each dose; therefore differences in exposure paradigm are an important factor contributing to these discrepancies (Kalant, 1996). The aforementioned studies used doses that ranged from 0.5 to 5.0g/kg with metabolic tolerance only present with the greatest dose after the fifth day of dosing from PN30 to PN50 every 48 hours. Repeated alcohol exposure in adults but not adolescents resulted in tolerance, as shown by naïve animals reaching significantly higher BEC than alcohol-exposed animals. In contrast to other studies relating to normal age-related BEC changes (Silveri and Spear, 2000), the BEC of tolerant adult animals was not significantly different to the baseline BEC of equivalent adolescent animals. Many factors
determine the level of response to alcohol, with age of initiation of treatment and age at which tolerance is determined, both being very important. Blood ethanol concentration is affected by age (Little et al., 1996; Morales et al., 2011; Walker and Ehlers, 2009; White et al., 2002) and also by the dose of alcohol used at the beginning of exposure (Broadwater et al., 2011).

The finding in the current study that BEC at four hours decreased significantly with repeated exposure is important, despite the lack of evidence of metabolic tolerance. Rats in the Silveri and Spear (2001) study did not show a progressive decline in daily peak BEC and this is likely because of the relatively low dose of alcohol of 2.6 g/kg that was used. The present study used a dose of 9.0 g/kg and used a different method of delivery. The concentration of alcohol and the route of administration, which was different in these studies, affect the BEC profile of both adolescent and adult animals (Walker and Ehlers, 2009). It is likely that the increasing age of animals with increasing days of exposure may account for the significant decrease in four-hour BEC in the present study. Blood ethanol concentrations change throughout development with the highest BECs being noted in adolescent animals and this would agree with the decrease observed over time being a decrease related to increasing age (Broadwater et al., 2011; Silveri and Spear, 2000). Although it was not
possible to develop extensive BEC profiles for every rat on every day in this study due to cost, it is possible that the time of peak BEC in response to the 9 g/kg dose may have occurred at different times depending on the animals’ age. Age and method of delivery also interact to produce changes in peak BEC with acute delivery of alcohol via i.p. injection in adolescent rats producing peak BEC faster than in adult animals (Little et al., 1996). Furthermore, pharmacokinetics of ethanol varies between rat strains (Erickson, 1984) and the use of Long-Evans in the present study rather than the more commonly used Sprague-Dawley rat may have influenced the BEC results. More data from adolescent human studies of the typical BEC profiles during binge drinking are needed to develop more reliable animal models.

2.3.4 Animal Models Currently in Use

To effectively model a uniquely human condition, such as adolescent binge drinking, in the animal, it is important to ensure that the model is designed to be as relevant as possible. In the current experiment we use intra-gastric gavage to deliver alcohol to the rat, at levels we had ascertained to be physiologically relevant, as absorption of alcohol via the digestive system mimics human alcohol drinking.
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It is important to note that intra-gastric delivery of alcohol is not the only means of delivery of alcohol used by researchers in the field, nor is it even the most popular. As the means of delivery affects peak blood ethanol concentration, rate of intoxication and rate of alcohol metabolism, primarily due to the absorption characteristics of alcohol in different tissues, it is an important aspect of model design. Whereas intra-gastric and vapour delivery can cause a slow onset of inebriation over several hours (Schulteis et al., 2008), i.p. injection can result in loss of the righting reflex in less than a minute (Pascual et al., 2007). Despite this major discrepancy to the way a human adolescent drinks alcohol, intraperitoneal injection is the most commonly used method of alcohol delivery in animal model research on the effects of adolescent alcohol exposure.

Currently, the most common paradigms are as follows: 5.0g/kg i.p. every 48 hours from PN30 to PN50 (White et al., 2000; Silvers et al., 2003; Silvers et al., 2006); 5.0g/kg intra-gastric with ‘top-up’ every 8 hours (approximately 9-10g/kg/day) PN35 to PN39 (Majchrowitz et al., 1975; Crews et al., 2000); 3.0g/kg i.p. every 24 hours from PN25 to PN38 with 2 days of dosing followed by 2 days of abstinence (Pascual et al., 2007); and 10 hours ethanol vapour for 3 consecutive days per week from PN32-34 to PN60-62 (Schulteis et al., 2008). There are of course many other exposure paradigms in use but for
the sake of brevity, I have used only examples involving repeated exposure paradigms and adolescent animals to allow comparison of models with the current study.

Comparison of doses between different experimental models can be difficult. A dose of 3.0g/kg given to PN30 or PN90 rats via i.p. injection or intra-gastric gavage resulted in significantly different blood alcohol profiles, with i.p. injection resulting in higher overall BECs and a faster increase in BEC (Walker & Ehlers, 2009). In comparison, the Crews et al. (2000) gavage model and Schulteis et al., (2008) vapour inhalation model involve multiple doses to maintain a target level of intoxication and thus animals will receive different doses. These differences mean that it is important to record measures such as BEC and BIS, to allow direct comparison between studies.

The different models currently in use all have advantages and disadvantages as a result of compromises in design. The two main models that use intraperitoneal delivery of alcohol fail to mimic the slowly increasing BECs that are typical for a human adolescent’s binge session. Instead, the entire dose is given at once and it is rapidly absorbed from the peritoneum reaching peak concentration in the blood at 15-30 minutes (Pascual et al., 2007; Walker & Ehlers, 2009). It is unknown whether this rapid increase in BEC results in
differential damage than the same dose would if it were delivered as smaller doses but it is likely, considering that evidence suggests that neurons respond differently to rapid increases in BEC regardless of the overall dose (White, 2003). Furthermore, a peak of 210±11mg/dL (Pascual et al., 2007) is relatively low, considering chronic alcohol users may have routine BECs around 300mg/dL (Urso et al., 1981).

The most commonly cited model that uses intra-gastric delivery of alcohol was initially designed to create physical dependence and gene induction in adult rats as well as reliably produce alcohol-induced brain damage. Its application to adolescent rats has allowed the differences in vulnerabilities between the adolescent and adult brain to be studied, and while the dose results in BECs of 557±17mg/dL (Crews et al., 2000), which are at the high end of what adolescents are likely experiencing, constant intoxication at that level for four days represents an unlikely scenario in a human adolescent. Furthermore, this model does not allow the multiple rounds of ethanol withdrawal that one would expect in the human adolescent binge drinker.

Vapour inhalation uses vaporised ethanol mixed with air as a method of delivery into the lungs and therefore the blood stream. This method of delivery has the advantage of being non-invasive, causing less exposure to stressful stimuli and it gives a slow increase
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in BEC that peaks at approximately four hours at 200-350 mg/dL depending on developmental stage. While, this magnitude of intoxication and rate of increase are relevant to a binge-drinking adolescent, the method of absorption is not and may have longer-term implications for the tissues of the respiratory system after repeated dosing.

In the current experiment, four equal doses of alcohol were given, 15 minutes apart to give a total dose of 9.0g/kg/day. Mean BEC for all treatment days at four hours was 276.9±9.3mg/dL and mean BEC on the first day of treatment at four hours was 338.2±23.1 (see Figure 2.4). These represent physiologically relevant ethanol concentrations and mimic what is recorded in binge drinking humans (Urso et al., 1981). Furthermore, the use of a large dose that is given over the course of 45 minutes is more reflective of the slow onset of inebriation that we are attempting to model. By allowing several days of abstinence between alcohol treatments we mimicked the weekly binge drinking of humans whilst still exposing the brain to multiple binges during the adolescent developmental window. This also allowed repeated ethanol withdrawal to become a potential factor in brain damage. The age range was carefully chosen to mimic mid-adolescence in humans and therefore the period of frontal cortical development mentioned above. Other important studies use
an earlier (Pascual et al., 2007) or later (Crews et al., 2000) age to start dosing but these models aim to encompass early and late adolescence respectively. As the Crews et al. (2007) model utilises an indwelling catheter, the stress response associated with an aversive gavage is avoided. In the current study, this may have caused a stress response as will be discussed below.

2.3.2 Stress Considerations in Gavage Treatment

Stress is an unavoidable component in this type of research and can be managed but not eliminated. The gavage procedure is invasive but alcohol delivery into the stomach is the most relevant form of alcohol administration. It is likely that the discomfort produced by gavage was not entirely due to the tube itself but by delivery of a relatively large bolus of alcohol into the stomach with consequent distension. This is an unfortunate result of alcohol being a drug that must be used in large volumes. Concentration of the alcohol solution was not increased as the absorption characteristics change markedly and this would not reflect absorption and thus the BEC profile of an alcohol-consuming adolescent during a binge session. Halothane was trialled as a short-term anaesthetic to lessen the potential stress response on dosing days. Unfortunately, this was
abandoned as it was found to increase the misintubation mortality rate.
3   Experiment Two
3.1 Methods

3.1.1 Rat Perfusion and Dissection

Approximately 24-hours after the final experimental treatment, animals were deeply anaesthetised using an intraperitoneal injection of sodium pentobarbital (60mg/kg IP) (Pentobarb, INJ, Animal Welfare Office, University of Otago). When the withdrawal reflex\(^2\) was absent the animal was placed on its back and limbs were stabilised on the perfusion rack with masking tape. An initial incision in the lower abdominal cavity was extended to open the chest cavity by lines of incision on along the diaphragmatic edge and both lateral edges of the cavity. This flap of ventral body wall was directed back over the head and held in place by artery clamp forceps, the pericardium was opened thus exposing the heart. The right auricle was cut to allow blood and perfusate outflow and a needle perfusion cannula was pierced into the tip of the left ventricle. The animal was trans-cardially perfused using a peristaltic pump, set on a pressure that gave a pulsatile outflow from the perfusion needle of 2cm on maximum flow. This measure was used as the standard pressure as the perfusion cannula and hence the

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\(^2\) When the animal does not retract its paw when a digit is squeezed with forceps, it has lost the withdrawal reflex.
pressure setting on the peristaltic pump changed as animals grew during the experimental period. Initially a pre-wash solution of 0.1M phosphate buffered saline with heparin (1xPBS PH=7.4) was pumped into the circulatory system of the animal to clear the vessels of blood. After two minutes of perfusion with 1xPBS/heparin solution the animal was perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PH=7.4) for another eight minutes. After the body had begun to stiffen the perfusion cannula was moved so that it flowed directly into the ascending aorta. Previous work in this laboratory has shown that this technique produces better perfusion.

Once the animal was perfused the head was removed and the skin was dissected free from the dorsal aspect of the head, from the neck to the nose. The superior skull was removed using bone rongeurs. The meninges were cut away at the lateral aspect using fine scissors and the cranial nerves were severed. The brain was lifted from the head with a rounded spatula, cleaned of remaining meninges and placed in 4% w/v paraformaldehyde solution for post-fixation overnight. Brains were then transferred to cryoprotectant solution containing 30% w/v sucrose in 1x PBS. Once the brains had sunk to the bottom of the vial containing the sucrose solution they were removed and rinsed in 1x PBS before the forebrain was dissected away from the cerebellum and brain stem. The two pieces of tissue
were gently dried using a lint-free tissue, lightly coated in Sakura™ Tissue-Tek™ (Sakura, Japan) compound and rapidly frozen in powered dry ice. The brains were then wrapped in chilled tin foil, placed in sealed, labelled plastic bags and stored at -80°C until sectioning.

3.1.2 First Phase of Histology

3.1.2.1 Coronal Sectioning

A preliminary study was carried out using animals from each group and each sex at ages PN29, PN33, PN37, PN41, PN45 as well as PN49 to investigate possible apoptotic cell death, and gliosis 24 hours after the initial alcohol dose. It was expected that there would be different vulnerabilities at different stages of adolescent development. Note that animals aged PN29 were assessed 24 hours after a single binge alcohol exposure, the initial binge, but at increasing ages, animals would have experienced from 2 to 6 binges. Assessment of acute apoptotic cell death however, applied only to the damage resulting from the most recent binge, as removal of apoptotic cellular debris occurs within 24 to 48 hours in brain tissue (K Naidu, Honours Thesis, University of Otago). Prior to sectioning, all tissue blocks were given a blind code, to ensure all tissue was analysed without bias. Brains were removed from the -80°C freezer
in the evening and stored at -20°C overnight. This allows the large tissue blocks to equilibrate to -20°C, the optimal cutting temperature, prior to sectioning on a cryostat (Lieca, Germany). Left hemispheres of these animals were sectioned coronally on the cryostat at 40µm and sections were collected in a random systematic sampling manner through the entire forebrain, so that many different brain regions could be examined. At each sampling point, three sections were collected and kept per well in 12 well BD Falcon cell culture plates (BD Biosciences, NJ, United States) containing cryoprotectant. A further three sections were cut and discarded and sampling repeated.

3.1.2.2 Early Immunohistochemistry and Apoptosis Survey

Sections were stored in cryoprotectant at -20°C and one section from each well was transferred to a custom made ‘insert’ (a total of four sections per insert) that fit into a well in a 24 well BD Falcon cell culture plate. ‘Inserts’ were 1cm long plastic cylinders of 1.5cm diameter with fine plastic mesh covering one end and allowed four sections at a time to be transferred into a new plate to change solutions efficiently. These sections were rapidly submerged in distilled water followed by rinses with 1xPBS (PH=7.4) to remove the cryoprotectant solution. Sections were incubated in 1:10 heat
inactivated goat serum (HIGS), diluted in 1xPBS, for one hour at room temperature on a slowly moving shaker. This was used to block the antigens in the tissue with low binding affinity proteins, ensuring that only the higher-affinity binding antibodies would dislodge more weakly bound proteins and attach to the specific antigens in the tissue. This also serves to decrease unwanted background staining by blocking sites where the primary antibody may bind non-specifically. Immediately after the blocking, sections were transferred into wells containing a small amount of primary antibody (see below). (A dilution series of a range of concentrations (1:200 – 1:1000) was carried out to determine the concentration that would provide optimal staining of this tissue). Sections were incubated at room temperature on a slowly moving shaker, for 24-hours before being washed three times in 1xPBS, blocked again for an hour and incubated for one and a half hours in the secondary antibody (1:2000) with attached fluorophore (Alexa Fluor 488 or 568, Thermo Fisher Scientific, MA, USA). Immunofluorescent labelling steps were completed in the dark so as not to exhaust fluorescence. Sections were washed again in 1xPBS, followed by 4xPBS and once more in 1xPBS. Sections were then incubated in Hoescht nuclear stain (Hoescht 33342, Invitrogen, Thermo Fisher Scientific, MA, USA) for five minutes. Hoescht stain labels double stranded DNA showing
normal nuclei and the condensed spherical bodies of nuclear material that are characteristic of a cell undergoing apoptosis. Sections were labelled with a primary antibody to NeuN (mouse monoclonal, clone A60, #MAB377, EMD Millipore, MA, USA) for labelling the nucleus and cytoplasm of neurons or GFAP (Glial Fibrillary Acidic Protein) (mouse monoclonal, G3893, Sigma-Aldrich, MO, USA) for labelling the cell bodies and processes of astrocytes. NeuN was visualized with an anti-mouse IgG, (Alexa 568, A-11019, Thermo Fisher Scientific, MA, USA) and GFAP was visualised with anti-mouse IgG, (Alexa 488, A-11017, Thermo Fisher Scientific, MA, USA). As both antibodies were monoclonal mouse antibody, staining for apoptotic neurons and glial cells was carried out on different sections. Sections were assessed in the fluorescence microscope to determine the presence of apoptotic nuclei in a number of key brain regions, including the anterior cingulate cortex, hippocampus and entorhinal cortex. Results are described below in section 3.2.1. However, the hypothesis that “acute apoptotic cell death will occur within 24 hours of binge exposure to ethanol” was not supported and led to the development of additional methods to further investigate this tissue as described below.
3.1.3 **Second Phase of Histology**

3.1.3.1 Production of Adolescent Rat Brain Atlas in the Coronal Plane

The digital version of *The Rat Brain in Stereotaxic Coordinates Fifth Edition* by George Paxinos and Charles Watson (2004) was used to delineate structures to be studied, however stereotaxic coordinates in this axis are based on a larger adult brain. Therefore, these coordinates did not allow for even approximate estimates of the spacing of key brain regions when developing a sectioning protocol. This was also necessary, as in the human, brain regions mature and reach adult size at different times during adolescence (Giorgio et al., 2010) and evidence outlined in the discussion of this topic supports this. Serial sections, three or four per slide, were mounted and stained with 0.025% acidified cresyl violet solution as per the staining protocol (see staining protocol: Appendix E) followed by differentiation with acidified 70% and 95% ethanol. Sections were dehydrated, cleared in xylene and coverslipped with DPX mountant (Sigma-Aldrich, MO, USA).

The atlas made for this experiment used a male rat brain at PN49; cut at 40µm with every third section, beginning with the first section to incorporate the orbital cortex, used. The other areas of interest
(AOI) were the rostral cingulate cortex, the dentate gyrus and CA1 regions of the hippocampus, and the amygdaloid nucleus.

Unfortunately, the existing equipment (Cast Grid, Olympus Life Sciences, Japan) failed and the newly purchased stereology system, a software package to be used on a conventional brightfield microscope, StereoInvestigator™, (StereoInvestigator v1.1, MicroBrightField Inc., VT, USA; Olympus BX51 light microscope, Olympus Life Sciences, Japan) was still to be delivered. There was considerable time delay prior to undertaking stereological assessment of the tissue and therefore was limited to analysis of the rostral cingulate cortex and the dentate gyrus/CA1 region of the hippocampus.

3.1.3.2 Histological Investigations

Prior to sectioning, the right hemispheres of PN49 rats (n=17) were equilibrated at -20ºC overnight, mounted, with the caudal surface lower-most, on a cooled cryostat chuk using Tissue-Tek™ (Sakura, Japan) and trimmed until the first section through the orbital cortex was obtained. The tissue was then sectioned at 40µm in the coronal plane and sections were placed in order in 12 well BD Falcon (BD Biosciences, NJ, USA) cell culture plates containing cryoprotectant, with five consecutive sections from the same location
per well. Sectioning continued along the rostral caudal extent until the orbital cortex and the most caudal part of the hippocampus had been sectioned. The section sampling protocol, designed with 7-8 systematic ‘stops’ and collection of five sections per stop within each AOI maximised the stereological sampling efficiency. Thus depending on the size of each AOI, the number of sections between sampling ‘stops’ differed. The actual sections taken throughout the brain are detailed in Appendix F. These sections were stored at -20°C prior to staining and subsequent analysis.

3.1.3.3 Immunohistochemical Labelling in Curved-Bottom Well Plates

After storage at -20°C sections were removed from cryoprotectant and placed directly into distilled water in wells of a plastic well plate (with holes as described). These well plates consisted of 36 curved-bottom wells in a 6x6 grid with each well having a maximum volume of 700µL. Optimal solution volume in these wells was between 200 and 500µL. Two variations of these trays were used; one tray had intact wells and was used for solutions that had to be used sparingly, such as the antibody solutions while the other had very small holes in the bottom and sides of the wells to allow solutions to drain in and out (see Figure 3.1).
This allowed the well tray to be placed inside a flat porcelain dish containing wash solutions enabling solution changes to be more efficient therefore more sections could be processed at once. It also importantly, reduced handling of the tissue sections and thus potential damage to the sections.

Sections underwent a two-second wash in distilled water to dissolve cryoprotectant and Tissue-Tek™ compound. Trays were
then transferred into 1xPBS for five minutes at room temperature on a slowly moving shaker. Sections were washed in 1xPBS a further two times for five minutes each, then transferred into 1% Hydrogen Peroxide (P289132, SigmaAldrich, MO, USA) for one hour at room temperature on a slowly moving shaker. Hydrogen peroxide was used to dissolve any remaining cryoprotectant; make minute holes in the cell membranes aiding antibody penetration; and destroy any endogenous peroxidases that would interfere with visualisation of the primary antibody using a horseradish peroxidase (HRP) conjugated secondary antibody. Three 1xPBS washes, of 10 minutes each, were followed by incubation in 1:10 HIGS in 1xPBS with 1:400 Tween-20 (P9416, Sigma-Aldrich, MO, USA) for one and a half hours at room temperature. Tissue was then incubated with the monoclonal mouse anti-rat GFAP antibody (mouse monoclonal, G3893, Sigma-Aldrich, MO, USA) at 1:400 in 1xPBS with Tween-20 for 20 hours at room temperature with gentle agitation. The following day, the sections were given three five-minute washes in 1xPBS prior to incubation in HIGS blocking solution for one hour. Sections were then incubated in a biotinylated, HRP-conjugated goat anti-mouse IgG, (A4416, Sigma-Aldrich, MO, USA) at a concentration of 1:1000 in 1xPBS with Tween 20 for one and a half hours.
Chapter Three: Experiment Two

This was followed with three 10 minute washes 1xPBS, a 4xPBS 10-minute wash and a 1xPBS 10-minute wash. The tissue was then incubated in 3,3’-Diaminobenzidine (DAB) solution (SIGMAFAST DAB, Sigma-Aldrich, MO, USA) for seven minutes. DAB solution was mixed immediately prior to incubation to prevent degradation of hydrogen peroxide in UV light.

Negative control tissue sections were included in each immune run where the primary antibody was omitted and all other procedures were identical. Positive control sections, sections from the adult rat brain (Dr Ping Liu), were included as they have an established astroglial cell population and were used to show the specificity of the GFAP primary antibody. There was very little in the published literature on the distribution of astrocytes in the early adolescent rat brain so the inclusion of positive control tissue was essential.

Sections were mounted on slides from 1xPBS and dried in front of a desk fan until just binding to the slide. The experimenter judged this when the PBS on the slide had dried around the section but the section was still moist. If sections are allowed to dry too much they can be prone to holding air bubbles, which interferes with histological examination.
Slides were counterstained in acidified 0.025% Cresyl violet for 25 minutes, dehydrated in a series of graded alcohols, immersed in xylene, mounted with DPX and stored in a dark place until analysis.

3.1.3.4 Microglial Marker Isolectin B4

A staining protocol was developed to label sections with a microglial marker isolectin B4 (BSI-B4, peroxidase conjugate, #L5391, Sigma-Aldrich, MO, USA). This protocol was based on previous work that used isolectin B4 to stain microglia within the central nervous system in both rats and mice (Ayoub and Salm, 2003; Depino et al., 2003; Thomas et al., 2004). However, despite numerous trials, we were unable to selectively stain microglia, as per the studies above, isolectin B4. Nonspecific staining was seen throughout the tissue in a seemingly random manner and with this uncertainty, stereology and quantitative analysis was not undertaken in this study. We concluded that as previous studies used freshly fixed tissue, the storage in cryoprotectant may have caused the nonspecific staining.

3.1.4 Stereological Methods

Stereological analysis was carried out on sections with the optical fractionator method using the StereoInvestigator™
(StereoInvestigator v1.1, MicroBrightField Bioscience, VT, USA) software system on an Olympus microscope (Olympus BX51, Olympus Life Sciences, Japan). The sampling parameters used for the optical fractionator methods are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Guard zones</th>
<th>1.50µm</th>
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<tbody>
<tr>
<td>Disector Height Z</td>
<td>7.00µm</td>
</tr>
<tr>
<td>Disector X</td>
<td>60.00µm</td>
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<tr>
<td>Disector Y</td>
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<tr>
<td>Frame area XY</td>
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<tr>
<td>Disector volume XYZ</td>
<td>25200µm³</td>
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<td>Grid X</td>
<td>185µm</td>
</tr>
<tr>
<td>Grid Y</td>
<td>200µm</td>
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</table>

Table 3.1  The above table shows the values used for the optical fractionator method in the current experiment.

The border of the area of interest was traced under the 1.25x objective lens, using landmarks and delineations as seen in a representative section from the rat brain atlas (Paxinos and Watson, 2004). The 60x objective lens was for counting within the optical fractionator.
The first unbiased sampling frame was applied at random to the delineated area of interest and all subsequent frames were applied in a systematic manner ensuring that all sampling was random systematic. Frames were included if over 50% of the area was within the outlined area of interest. All cells within the 60x60µm unbiased sampling frame were counted except those whose cell body touched or crossed an exclusion line or exclusion corner, through a measured depth within the section. For GFAP labelled sections two counts were made; labelled cells with less than four branches leaving the cell body were counted separately to those with four or more branches leaving the cell body. The total number of cells, irrespective of branch number was calculated. All branches had to be seen leaving the cell body for them to be included in the count for that cell (see Figure 3.2).
An estimate of the total number of cells in the area of interest was determined using the following equation with counts obtained from the 3 representative sections.

\[ N = \frac{1}{ssf} \times \frac{1}{asf} \times \frac{1}{hsf} \times Q \]
Where ssf equals the section sampling fraction (= 3/111), asf equals the area sampling fraction (= 3600/37000) and hsf equals the height of the section within which cells were sampled (= 7µm/the mean section thickness in µm across all counting frames on the section).

Unfortunately some brains were poorly preserved and the tissue degraded during the period between the failure of the first stereology microscope and the availability of the second, resulting in exclusion of tissue and the treatment groups no longer having an even balance of male and female rats. Results for this part of the study are presented but are interpreted in the light of a potential gender bias existing within the treatment groups as follows: Control: 4M, 2F; Ethanol-treated: 1M, 5F.

3.2 Results

3.2.1 Ethanol-Induced Apoptotic Cell Death and Changes in GFAP Labelling

A representative micrograph from a female control animal aged PN33 is shown in Figure 3.3.
The spherical units of condensed nuclear chromatin, characteristic of an apoptotic cell, can be seen in Hoescht labelled material as a very bright fluorescent spot or cluster of spots, as can be seen in the above micrograph (Figure 3.3). Some apoptotic cell death was present but occurrence was infrequent. Survey of a large number of sections from different rostro-caudal positions across all groups and ages found only a small number of apoptotic cells in any one section,
irrespective of treatment or location and thus cell number was not quantified.

The overall pattern of immunolabelling for GFAP was low in PN29 animals of both treatment groups. GFAP labelled astrocytes were found in the white matter and in the outermost zone of the cortex indicative of astrocytes forming a layer immediately adjacent to the pial membrane and blood vessels, however, there was very little GFAP label in the cortical grey matter structures. No qualitative analysis was performed on PN29 tissue. Representative PN49 sections containing selected brain regions were selected, coded and each region of interest was photographed. Within each region, photographs were placed in order of the density of GFAP labelling to determine if there was a qualitative difference in appearance across treatment group. There appeared to be no obvious qualitative relationship between treatment group and GFAP staining in the older PN49 animals in most of the regions photographed. However, GFAP staining in the cingulate cortex did appear to be more pronounced in the alcohol treated animals. Thus the cingulate cortex was selected for further investigation in the second phase of histology (see Section 3.2.3) with the methods for this second phase found in Sections 3.1.3.2 to 3.1.4.
3.2.2 Qualitative Analysis of Dentate Gyrus and CA1 Region of the Hippocampus

Unfortunately, the dentate gyrus and CA1 region were not analysed using stereology because the small cell bodies of the astrocytes could not be reliably distinguished from the background of extensively DAB-stained GFAP-labelled astrocytic branches. Staining in these regions was highly variable, with some animals displaying astrocytes with hypertrophied and thickened branches that were clustered around blood vessels and pervading the hippocampus (see Figure 3.5).
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Other animals showed many fewer astrocytic branches that were less complex with each branch a finer process. As these different appearances occurred in sections processed in the same run of immunohistochemistry, the differences could not be attributed to differences in the execution of the immuno-labelling protocol. All treatment groups showed a similar variation of staining, so these differences were not likely caused by treatment.

Figure 3.5 The two figures above show the extensive variation that was observed between GFAP labelling in in the region of the dentate gyrus and CA1 in (A) a PN49 ethanol treated female and (B) a PN49 control intubated female. Note that these animals underwent immunohistochemistry in the same run.
3.2.3  **Stereological Estimate of Astrocytes in the Rostral Cingulate Gyrus**

An unpaired t-test revealed there were significantly less astrocytes with four or more processes leaving the cell body in the alcohol-treated group, compared to the control group \( t(10) = 2.405, p = 0.0370 \) (see Figure 3.6).

![Figure 3.6](image)

**Figure 3.6**  The above figure shows the estimated total number of 4+ branch GFAP-labelled cells in the rostral cingulate gyrus of animals aged PN49.

There was no significant difference between treatment groups in the total number of astrocytes with less than four processes leaving the cell body \( t(10) = 0.006566, p = 0.9949 \).
The total number of astrocytes in the rostral cingulate cortex was significantly less in the alcohol-treated animals compared to controls \([t(10)=2.252, p=0.0480]\) (see Figure 3.7).

![Figure 3.7](image)

**Figure 3.7** The above figure shows the significantly reduced estimated total GFAP-labelled cell counts for the rostral cingulate cortex.

As outlined in the methods (3.1.4), these results may have been influenced by a gender bias across the treatment groups and will be addressed in the discussion of these results.
3.3 Discussion

3.3.1 Apoptosis Survey

The main finding of experiment two was a lack of any increase in the number of apoptotic cells in the brains of alcohol-treated animals 24 hours after each period of alcohol exposure. This did not support the hypothesis that adolescent binge drinking would cause apoptotic cell death in the brain of rats within 24 hours of alcohol exposure. This was very surprising, as extensive apoptotic cell death has been demonstrated in various rodent models of adolescent binge drinking (Crews et al., 2000; Oliveira-da-Silva et al., 2009; Pascual et al., 2007). Neuronal cell death, visualised by positive staining with silver cupric stain, was found in the olfactory bulbs, frontal cortical olfactory regions and the anterior piriform and perirhinal cortices of adolescent rats one hour after the cessation of alcohol administration (Crews et al., 2000). However, it is important to consider that the model used by Crews et al., (2000) was initially developed to reliably produce alcohol-induced brain damage (Oberniera et al., 2002). Their method results in moderate but continuous intoxication over a period of four days. Although it was found that cell death was more severe in certain regions in the adolescent compared to the adult brain, it is difficult to equate this regimen to binge drinking in
human youth (Kypri et al., 2002; de Bonnaire et al., 2004; Kypri et al., 2009). Pascual et al. (2007) demonstrated the presence of apoptotic cell death in the alcohol-exposed adolescent brain using Western blot analysis to show significantly greater amounts of activated caspase-3 (a key effector enzyme in the apoptotic cascade) and DNA laddering (a specific pattern of DNA fragmentation characteristic of DNA breakdown in apoptosis) in the neocortex, hippocampus and cerebellum. Importantly in this study there was daily ethanol administration, via intraperitoneal injection, on each of 2 consecutive days followed by 2 alcohol-free days for the duration of adolescence, PN25 to PN38. A similar study in mice using i.p. injection of ethanol on alternate days from PN30 to PN45, found a significant increase in TUNEL-positive cells throughout the hippocampus 24 hours after the cessation of ethanol treatment (Oliveira-da-Silva et al, 2009). The key variable here is that the method of delivery, i.p. injection of alcohol, resulted in a very rapid increase in BEC which may result in a different cellular response than the more gradual increase that was observed in the current study.

Considering the above data it was very surprising that the current study did not replicate these findings and there may be a number of reasons for this. Firstly, if the cell death reported by other research
groups is caused by excitotoxicity, perhaps continuous intoxication for more than one day is required to elicit a change in NMDA receptor subunit composition, as has been demonstrated in *in vitro* studies (Smothers et al., 1997). Secondly, the time-point used in the current study to assess apoptotic cell death was 24 hours after each ethanol delivery, as in the previous studies (Crews et al., 2000; Oliveira-da-Silva et al., 2009; Pascual et al., 2007). This would only provide evidence of apoptotic cell death from the most recent exposure but was in line with previous studies; and prior work in this study had shown a single binge exposure alone on PN29 did not elicit acute cell death. The specifics of the alcohol exposure paradigms used varied between studies and may have been crucial in eliciting acute apoptotic cell death. The present study used a paradigm that resulted in high blood ethanol levels, and although hospital emergency facilities report blood ethanol levels this high in youth, many of which have been brought to hospital due to being found unconscious, there is no accompanying data on the pattern of drinking that elicited such blood ethanol levels (Bouthoorn et al., 2011; Langhan, 2013; Zanten et al., 2013). It is most likely though that these youth experience considerable delay between the initiation of drinking and achieving coma-inducing levels of intoxication and that they undertake this behaviour on a single day followed by days of
abstinence (Bouthoorn et al., 2011; Langhan, 2013; Zanten, 2013). This is pertinent as intoxication in the Crews et al. model (2000) extended for 4 days, intoxication for a continual 96 hours, a very unlikely scenario in humans. This does not discount the interesting difference in tissue response between the adolescent and adult brain but it does suggest it is not a viable model for adolescent alcohol exposure. The temporal delivery of ethanol by Pascual et al. (2007), a single dose of ethanol on 2 successive days followed by 2 alcohol-free days throughout adolescence, does represent a viable model of the pattern of youth drinking. The condensed on and off cycle over 4 days, to accommodate the short period of rat adolescence, may not equate reliably to human adolescence but the different length of the life cycle of rodents versus humans requires a more condensed delivery regime. However the alcohol in this model was given via i.p. injection and resulted in the loss of the righting reflex within 96 ± 14 seconds indicating that ethanol was delivered very rapidly to the brain, causing almost immediate sedation. This may cause a very different cellular response than a gradual increase to peak BAC as used in the present study and is worthy of further investigation. Many adolescent alcohol models deliver ethanol via i.p. injection and, as 20% ethanol is regarded as tissue fixative, as well as the very rapid delivery of alcohol into the blood stream from the very
vascular peritoneum, this can not be considered as an ideal method with which to simulate adolescent alcohol exposure. Genetics can confer susceptibility to certain toxins and rodent species and rat strain may be an important component of the variability within the published data (Kacew, 1996). Previous work has used Sprague-Dawley rats (Crews et al., 2000), Wistar rats (Pascual et al., 2007) and C56BL/6J mice (Oliveira-da-Silva et al, 2009) known to be sensitive to ethanol, whereas the current study used Long-Evans rats (Crabbe et al., 1999).

Blood ethanol concentration has been shown to be a crucial variable in eliciting damage in the developing brain and as the adolescent brain is undergoing considerable remodelling and synaptic stabilization it was considered that BEC may again be a crucial determining factor (Becker, 1997). The present study aimed for a high but not extreme peak blood alcohol level, as seen in adolescent hospital admissions, but did not elicit acute apoptotic cell death whereas peak BECs of 211 ± 11 mg/dL (Pascual et al., 2007) and 137.2 ± 8.5mg/dL (Oliveira-da-Silva et al, 2009) did elicit apoptotic cell death.

Clearly there needs to be more work done to clarify whether ethanol does elicit apoptotic cell death in the adolescent brain, an important issue as cell death occurring in adolescence would likely
result in permanent cell deficits in key brain regions such as the CA1 region of the hippocampus. It is essential however, that physiologically relevant models be used to investigate the real impact of ethanol as a neurotoxin in the adolescent brain as data from a human longitudinal study has shown a significant increase in negative life outcome when alcohol consumption began prior to 15 years of age (Odgers et al., 2008).

3.3.3 Stereology in the Rostral Cingulate Gyrus and GFAP Survey

As stated previously, GFAP immunoreactivity in various brain areas were examined after exposure to alcohol. The cingulate cortex and hippocampus were chosen as areas of interest based on qualitative differences in GFAP expression in early experimentation and current literature. There has been study of the effects of alcohol on astrocytes in several brain regions including the hippocampus, but not the cingulate cortex. In the current study, GFAP-labelled cells were fewer in the rostral cingulate cortices of ethanol treated animals.

As stated in the methods and results, treatment groups did not have equal numbers of male and female animals and this may affect
results. If gender were the cause of the lower GFAP-stained cell count it would follow that male animals would have a greater number of astrocytes in the cingulate cortex, compared to females. Unfortunately, it would not seem that the sexual dimorphism of astrocytes in all regions of the rat brain has been studied, as evidenced by a lack of published literature on this topic. As oestrogen is neuroprotective, many studies use either male or ovariectomised females to control for oestrogen concentration during the oestrous cycle. When both sexes are used, they are normally balanced and few studies investigate the actual sex differences within the data. As stated, the cingulate cortex has not been studied, however associated brain regions have. One particular study that investigated the prefrontal cortex for astrocytic expression of basic fibroblast growth factor (bFGF or FGF-2) in response to ovariectomy showed that ovariectomised rats given control treatment or a synthetic oestrogen showed no significant difference in concentration of marker (Flores et al., 1999). While this study looks at the concentration of the actual response of the astrocyte rather than the concentration of the astrocytes themselves in this brain region it is supporting evidence that oestrogen does not account for a difference in astrocyte morphology in the prefrontal cortex (Flores et al, 1999). Similarly, when astrocytes were labelled using GFAP in the
dorsal raphe, which projects serotonergic neurons to the brain, and ventral tegmental area, which is a key projection area to the prefrontal cortex, there was no difference between control ovariectomised rats and ovariectomised rats given synthetic oestrogen (Flores et al., 1999). With respect to GFAP expression, specific brain regions appear sexually dimorphic. For example, in rats the hippocampus contains more GFAP-positive astrocytes in the male than in the female (Hur et al., 1999). When subregions were investigated using stereology, it was found that males have a greater number of GFAP-expressing cells in CA3, whereas females had a greater number in CA1 (Conejo et al., 2003). In adolescent animals, there were more GFAP-expressing cells in males in both of these subregions (Conejo et al., 2005) however, other studies have concluded that the female mouse hippocampus contains a greater number of GFAP-positive astrocytes regardless of age range used compared to age matched males (Mouton et al., 2002). In the rat amygdala, males have a significantly greater number of GFAP-expressing astrocytes than females in the right hemisphere only, with more complex astrocyte morphologies in the left hemisphere of this brain region (Johnson et al., 2008). These few examples from the literature suggest that sexual dimorphism in astrocytes can be variable depending on the age of the animal, the particular brain
region and the hemisphere being investigated, therefore this experiment should be revisited in future work to identify whether the results in the current study are actually a result of sexual dimorphism.

It was expected that alcohol would cause increased GFAP expression as per the results of Pascual et al. (2007). Hayes et al. (2013) investigated the effect of one to two days of alcohol on vimentin levels in the hippocampus and associated limbic cortex and reported reactive gliosis in the hippocampus but presumably not the limbic cortex. More interestingly and in support of the current study are the results of Koss et al. (2012) who reported a reduced number of astrocytes in the medial prefrontal cortex of male rats but not females after delivery of 3g/kg alcohol via ip injection in a ‘two-days on one-day off’ binge-type pattern. There was no reduction in neurons in this region in either males or females. It is likely that dose and timing will affect an astrocytic response as previously suggested for apoptosis.

Gliosis has often been seen as evidence of necrotic cell death (Switzer, 1993), as it accompanies the removal of cellular debris, however it is now thought that it can occur independently of
necrosis (Switzer, 1993; Clarke, 1999). A recent study demonstrates only partial activation of microglia, presenting [H]-PK-11195 and OX-42 and not OX-6 and ED-1 in response to a four-day alcohol binge that causes neurodegeneration (Marshall et al., 2013). Also present were anti-inflammatory cytokines and growth factors but not pro-inflammatory cytokines. Therefore in this instance, partial microglial activation occurred independently of neuroinflammation but occurred in the presence of neurodegeneration. In other studies, chronic alcohol exposure has caused gliosis in alcohol-treated animals (Alfonso-Loeches, 2013) but this also occurs in response to only 24-hours of elevated BEC (Hayes et al., 2013). Gliosis has also been shown throughout the brain, where cell death is also present, of adult male rats that were exposed to a four-day binge of alcohol, as evidenced by the FluoroJade B stain for neurodegeneration (Kelso et al., 2011). Thus the role of gliosis, inflammation and cell death in the adolescent brain in response to alcohol is still poorly understood.

It was thought, based on other research reporting extensive gliosis reactions to alcohol, that PN29 would be the most likely age to see treatment group-dependent gliosis in the current study. However, GFAP labelling was very limited in the PN29 animal as can be seen in Figure 3.8. This was unexpected as astrocytes develop throughout postnatal development and are distributed throughout both grey
and white matter. It is now thought that while there were astrocytes they were not mature to the point of expressing adult levels of GFAP. However other studies, which have used different rat strains, suggest GFAP-positive astrocytes become numerous at PN11 (Kalman & Ajtai, 2001) and GFAP reaches adult levels at around one month of age (Burette et al., 1998). Figure 3.9 is a figure from the Kalman & Ajtai paper for comparative purposes, however the strain of the rats used is not specified, so differences in developmental rate can only be suggested.
Figure 3.8  DAB labelling of GFAP in the superior portion of a rostral section of forebrain from a PN29 animal. Note the staining occurs at the periphery of the section and in the white matter.
Figure 3.9  GFAP labelling in the PN7 rat cortex, to demonstrate GFAP immunoreactivity (single arrowhead) in immature brain tissue (Kalman & Ajtai, 2001).
Some figures do resemble representative images of gliosis, which suggests that there may have been an insult that was causing GFAP proliferation (see Figures 3.10 and 3.11, which show control and alcohol-treated animals, respectively, from the current study). Figure 3.12 shows progression of gliosis after an insult, deafferentation of the optic nerve (Schmidt-Kastner et al., 1993). Some control animals that were not exposed to alcohol showed signs of this gliosis, however, so future studies would benefit from a more widespread

Figure 3.10: Appearance of GFAP-stained astrocytes in the anterior cingulate cortex of a PN49 alcohol-treated animal. The appearance of these thickened, highly branched astrocytes suggests presence of reactive-gliosis (see Figure 3.11 for control).
survey of glia.

Figure 3.11: Appearance of GFAP-stained astrocytes in the anterior cingulate cortex of a PN49 control animal. Note the astrocytes have thinner branches than the above image.
Figure 3.12  Immunoreactivity to GFAP increases after deafferentation of the superior colliculus at different time points (Schmidt-Kastner et al., 1993). Micrograph (A) shows control animal (B) 1 day (C) 2 days (D) 4 days (E) 8 days and (F) 21 days.
4 Experiment Three
4.1 Methods

4.1.1 Behavioural Apparatus

Behavioural experiments were conducted in Testing Room 3 of the BPU, a windowless, temperature and humidity controlled, soundproof room. Experiments were performed during the animals’ light cycle under wall mounted, white light (light intensity of 15 lux) unless otherwise stated. Noise and movement from the experimenter was kept to a minimum and the experimenter remained in the same location in the room during the video recording of a test. Objects around the room, visible from the rat’s position inside each maze were kept constant, as these would become distant visual cues. Rats were transported from their home cage across the room to the apparatus in a high-sided black plastic box with an area of 400mm by 400mm. A video camera was used to record the rats’ behaviour for offline analysis using the TopScan 2.0 software package (TopScan 2.0, Cleversys Inc™, USA). The camera was suspended above the maze on a rod supported by two tripods, at a height of 1800mm.

4.1.1.1 Open Field
The open field (OF) test used overhead fluorescent tube lights (light intensity of 609 lux) in addition to wall lighting. The apparatus was a large opaque plexiglass box measuring 600mm by 600mm with walls 300mm high, located in the centre of the room. The apparatus is shown in Figure 4.1.

![The open field apparatus, as used for open field and novel object testing.](image)
4.1.1.2 Novel Object Recognition

Novel object recognition (NOR) was conducted in the OF apparatus. Three objects were used, two pieces of plastic fruit and a small porcelain jar. One object was a capsicum approximately 5cm high, coloured dark green with a green stalk and a smooth texture; one was an orange approximately 5cm high, coloured orange with a green stalk and a textured surface; and one was a small porcelain jar approximately 4cm high attached open-end down (see Figure 4.2). Two objects were placed in a line running east-west through the centre of the box and attached firmly to the base with Blu Tak™ (Bostik, France), approximately 200mm from the east and west walls.

![Three objects](image_url)

Figure 4.2 The three objects used in the novel object recognition experiment.
4.1.1.3 Elevated T Maze

The elevated T-maze (ETM) apparatus was essentially an elevated plus maze, with access to one closed arm blocked. It was made of opaque plexiglass and was shaped like a plus sign with two arms having high sides (closed arms) and two arms not having high sides (open arms). The maze was elevated to 1500mm by a stainless steel frame to make the open arms more anxiogenic.

![Elevated T Maze Diagram](image)

Figure 4.3 The setup of the apparatus that was used for the elevated T-maze in the current experiment.

The dimensions of the apparatus were as follows: the arms were 500mm long and 100mm wide. The walls of the closed arms were
300mm high compared to the walls of the open arms that were 10mm high. The arms were all connected at the centre of the maze at a central area square measuring 100mm by 100mm. The maze was oriented so that the open arms were East and West and the closed arms were North and South (as per their relation to the image captured by the video camera). Figure 4.3 on the previous page shows a diagram of the elevated T-maze.

4.1.1.4 Morris Water Maze

The Morris water maze task (Morris, 1984) was performed in a black circular plastic tank measuring 1500mm in diameter and 450mm in height. A circular platform made of clear plexiglass measuring 100mm in diameter attached via a clear plexiglass tube to a black metal weight formed a platform 250mm high. The tank was filled with water to a height of 270mm (20 millimetres above the platform).

The water in the tank had approximately 250mL of full cream milk powder added to make the water opaque and thus obscure the platform and to make the swimming rat more visible to the camera and analysis software. Points around the pool were designated as North, East, South and West with North being the top of the camera
image. The platform was located in the Southwest quadrant for training trials and was moved to the Northeast quadrant for the reversal trials (see Figure 4.4).

![Figure 4.4](image)

**Figure 4.4** The setup of the Morris water maze showing platform locations.

### 4.1.2 Behavioural Procedures

#### 4.1.2.1 Open Field

Each trial of behavioural testing was recorded on video and analysed post-hoc. The video recording was started and the rat was transported to the apparatus. Each rat (n=26) was lifted by the tail
out of the holding box and lowered into the centre of the apparatus, facing the South East corner. The front paws were lowered to the floor and the back paws were allowed to drop, as this is thought to encourage the animals to explore immediately rather than scrabbling away from the experimenter (Deacon, 2006). This method of entry was performed for all experiments conducted in the open field apparatus. A timer was started as each animal was placed in the apparatus and they were allowed to explore for a period of 20 minutes while their movements were recorded. This was repeated the following day. Path length and duration in the inner and outer zone were analysed for each trial, using Topscan (Clever Sys Inc, 2010, USA). The first five minutes were analysed independently of the final 15 minutes because exploratory behaviour may only last a short time. Between trials the apparatus was cleaned using an ethanol/hibitane solution to remove olfactory cues left by the previous animal.

4.1.2.2 Novel Object Recognition

Novel object recognition (NOR) was conducted in the same apparatus as the OF test so that the OF test was used as habituation for the NOR apparatus. The test, consisting of four phases, was
conducted over two days with two phases of five minutes, approximately six hours apart, each day for each animal. The first phase of day 1 consisted of habituation to two objects, the two ‘fruit’ objects. Recording was started and the animal was placed between the two objects facing the South wall. After each phase the rat was removed to its home cage. The objects were removed and cleaned along with the apparatus, with ethanol/habitane solution. Object location was counterbalanced across groups and sexes for all phases. The location of the new object in phase four of the test was also counterbalanced across groups and sexes.

Phase two was a repeat of phase one to further habituate the rat to the fruit objects. The locations were kept constant from the first phase, as this experiment did not aim to test the animals’ detection of a misplaced object. On the second day, phase three was identical to phases one and two. In phase 4 the capsicum was replaced with the porcelain jar, the novel object. The novel object was the same size as the non-novel objects but was of different shape and colour. No object had any tight crevices that could hold smell that could be overlooked when cleaning and all objects used were made of materials that would not absorb smell or be easily chewed. After a pilot experiment to test rats’ behaviour with a variety of objects made of different materials, it was found that the rats showed
particular interest in objects that they could sit on, and wooden and other objects that could be chewed or could hold smell and these were not used in the test.

4.1.2.3 Elevated T-Maze

Figure 4.5 A rat completing the 30-minute habituation phase on the open arm.

Animals entered the testing room in their home cage and thus in their housing groups. Each animal was transferred to the holding box for the duration of the T-maze testing, which was cleaned
between animals. There were three parts to the elevated T-maze experiment: the habituation phase, the learned avoidance phase and the one-way escape phase. On the first day each animal underwent a habituation phase, where it was placed on an open arm for 30 minutes and its escape into the central area was blocked using a plexiglass divider (see previous page, Figure 4.5). Rat treatment groups and sexes were counterbalanced across the two opposite open arms and each rat performed the escape test starting on the same open arm that they were habituated on. Only one closed arm was used for all experiments. At the completion of each trial faeces were removed. The apparatus was cleaned with hibitane/ethanol solution between animals but not between trials.

To test learned avoidance, the rat was placed in the closed arm of the maze with access to both of the open arms. The recording was started as the rat was placed in the far end of the arm facing the middle of the maze and it was stopped when the rat had satisfied the criteria of exiting the arm, of having all 4 paws over a line that delineated the point at which the closed arm became the central area. The learned avoidance test consisted of three trials performed with a 30-second period between each trial in the holding box. In each trial the animal was allowed a maximum of 300 seconds to exit the closed
arm, after which time the animal was removed from the maze. The time to exit the closed arm was recorded and if the animal did not leave the closed arm, 300 seconds was recorded as the exit time. In this circumstance the rat did undertake all future trials. The escape test also consisted of three trials, each of 300-second duration, with an inter-trial interval of 30 seconds. The rat was placed in the far end of the open arm facing the middle of the maze with access to the other open arm, and the previously used closed arm. The animal had completed the task when all four paws had crossed over the line that delineated the boundary between the starting open arm and the central area. Computer analysis was performed on the recorded videos and latency to leave the arm was analysed for each trial.

4.1.2.4 Morris Water Maze - Reference Memory Version

Rats received four consecutive days of training with four trials per day and an inter-trial interval of 10 minutes. There were four starting locations; North, East, South and West with each used in a pseudo-random order per day, but such that the last location on any one day was never used as the first location on the following day. Rats were placed into the water with a one-handed hold around the body behind the forepaws, were released, facing the wall and were
allowed to swim for 60 seconds, or until they found the platform. After 60 seconds if the rat had not found the platform it was guided towards it with a short piece of wood. When the animals found the platform they climbed onto it and were allowed to remain for 10 seconds in order to observe visual cues and thus be able to orient to and find the platform in subsequent trials. The experimenter remained seated in a fixed position while the rat was swimming and on the platform, so as not to become a variable visual cue.

The animal was removed from the platform following each trial, towel-dried and placed in the holding box on top of a heating pad for five minutes before being returned to their home cage. Rats performed the trials in groups composed of their cage mates. The rats were tested in the same order each day, with each rat completing trial one in order before the first rat moved to the next start position to complete trial two and so on. On each subsequent day trial one began with the rat that was second in training order on the previous day, so that all rats would experience each position in the training sequence to allow for the potential confound that training order impacted on MWM performance.

To analyse the recorded videos with TopScan, the water maze was set up as an ‘arena’ with an inner area, an outer area (a 100mm wide circular band inside the outer edge of the tank), and the
platform zone. The total area of the maze, subdivided as the inner and outer area areas, was further divided into four quadrant zones (North East, South East, South West, North West). The path length in each zone was recorded as well as the rats’ latency to enter and time spent in each zone. The temperature of the water in the Morris water maze was 28 degrees, plus or minus 2 degrees Celcius.

4.1.2.5 Morris Water Maze Probe Test

Twenty-four hours after the last training day the rats performed a probe test, where the platform was removed and rats were allowed to search for 60 seconds. The data pertaining to the rats’ movements was analysed using Topscan in a similar way to the ‘training’ trials. Proximity to the platform zone was measured, which is a measure of focused searching determined by the distance from the centre of the rat to the centre of the platform zone, at every second, averaged for each second of the 60-second trial.

A Whishaw’s corridor zone was also used to determine how close the rat’s initial swim path is to the ideal path to the hidden platform. This is done by drawing a narrow corridor from the starting location to the platform location and recording how much of the rats’ initial path occurs within this corridor. The first five seconds of each probe
trial was analysed again separately, as the initial behaviour of the rat may be more indicative of memory than behaviour after discovery that the platform is not present at the expected location. Finding the platform is not in the ‘remembered’ location a rat may utilise an alternative strategy to search for an alternative platform location.

4.1.2.6 Morris Water Maze Reversal Training

To assess the animals’ ability to forget, or more accurately to unlearn, the first platform location and learn a new location, two days of reversal trials were conducted. The reversal trials began on the day following the probe trial. The new platform position for reversal trials was the Northeast quadrant and trials were carried out as per the training trials. The task was undertaken as per training in the reference memory version and the same parameters were analysed including the location of the old platform and the probe analysis parameters.

4.1.2.7 Morris Water Maze Search Strategy Analysis

Traditional measures of Morris water maze performance (e.g. path length, latency, duration in target quadrant) can be considered as one-dimensional measures. Spatial memory is undoubtedly very
complex so in the current study, we also analysed the specific search strategy used by each animal to investigate more subtle aspects of water maze behaviour that are not assessed by the single measures of time, distance or location. A literature search revealed that certain search strategies have been classified and their use by rodents in the MWM task has been studied (Brody and Holtzman, 2006; Graziano et al., 2003; Petrosini et al., 1996). The most relevant, in terms of experimental intervention, to the current experiment were search strategies determined by Petrosini et al. (1996) and Brody and Holtzman (2006) who used search strategy analysis to further highlight changes in MWM performance in response to traumatic brain injury (TBI). In the former, animals with cerebellar lesions show a preference for peripheral circling while in the latter, animals with genetically increased amyloid precursor protein (a protein associated with Alzheimer’s Disease) relied on non-spatial systematic strategies. It was noted that with training, animals in the latter study used spatial search strategies rather than repetitive looping. However after TBI, rats with increased amyloid precursor protein reverted back to repetitive looping while wild-type animals reduced their use of spatial strategies but relied instead on non-spatial systematic strategies.
The search strategies of Graziano et al. (2003) are very well defined and were used in the current study. Paths were assigned to one of seven search strategies: thigmotaxis (an animal’s tendency to move alongside a fixed object), circling, random searching, scanning, self-orienting, approaching target and direct finding as shown in Figure 4.5. Each trial of the training sessions, the probe trial and the reversal training trials the path followed by the animal was converted to a single representational image of the path followed within the water maze and then assigned to a search strategy. The proportionate use of each strategy within each trial was determined for each treatment group and analysed to assess the effect of treatment on search strategy.

4.2.1.8 Statistical Methods

All behavioural data was tested using two-way repeated measures analysis of variance (ANOVA) followed by a range of post-hoc tests as appropriate for the data. Some data had to be normalised, using appropriate statistical methods. The significance level was set at $P<0.05$ for all comparisons. Unpaired t-test was used for platform crossing in MWM data analysis. All calculations were
performed using GraphPad Prism 6 (GraphPad Prism 6, GraphPad Software Inc, USA).

Figure 4.5 Above is a diagram showing representative paths of rats performing the different search strategies while finding the platform in the Morris water maze (from Graziano et al., 2003).
4.2 Results

4.2.1 Open Field

The path length performance of treatment groups across both days of the open field test is shown in Figure 4.7. There was no significant effect of treatment \( [F(2,46)=0.2373, \ p=0.7897] \) or trial day \( [F(1,46)=3.980, \ p=0.0520] \) and no interaction between treatment and trial day \( [F(2,46)=0.2150, \ p=0.8074] \). Figure 4.8 represents these data as a scatter plot.

Control groups were combined to allow sex differences between treatments groups to be analysed.
Figure 4.7  Total path length (mean ± SEM) in the open field test for both days for all treatment groups. There was no significant effect of treatment, day and no interaction.
A two-way ANOVA revealed there was no effect of treatment on path length \( [F(1,22)=0.05593 \ p=0.8152] \) on day 1 in the open field, there was a significant effect of sex \( [F(1,22)=16.48, \ p=0.0005] \) but no interaction of sex and treatment \( [F(1,22)=0.01438 \ p=0.9056] \). Sidak’s post-test indicated there was a significant difference between male and female animals’ in path length on day 1 in both the alcohol group \( [p<0.05] \) and the combined control group \( [p<0.05] \) (see Figure 4.9).
A two-way ANOVA again indicated there was no effect of treatment on day 2 of open field testing \[ F(1,22)=0.9332 \ p=0.3445 \] but the significant effect of sex remained, \[ F(1,22)=25.51, \ p<0.0001 \] with no interaction of sex and treatment \[ F(1,22)=0.006436 \ p=0.9368 \]. Sidak’s post-test indicated there was a significant difference between male and female animals in both the alcohol group \[ p<0.01 \] and the control group \[ p<0.01 \] (see Figure 4.10).

Figure 4.9   Total path length on day 1 of the open field test, males compared to females. There was a significant effect of sex, within both treatment groups. M=male, F=female, E=alcohol, C=control * p<0.05
Behaviour in the open field is influenced by familiarity with the environment. To investigate the effect of familiarity influencing behaviour over time, for each of the two days, the open field test was divided into four five-minute blocks with each block analysed using two-way ANOVA (Figure 4.11).

On day 1 there was a significant effect, in the alcohol treated animals, of time block \([F(3,40)=26.78, \ p<0.0001]\) and of sex \([F(1,40)=13.69, \ p=0.0006]\], but there was no interaction \([F(3,40)=1.094, \ p=0.3628]\).
There was also a significant effect of sex \[F(1,48)=26.92, \; p<0.0001\] and time block \[F(3,48)=82.48, \; p<0.0001\] but no interaction on the combined control animals (see Figure 4.11).

On day 2, on the alcohol-treated animals, there was still a significant effect of time block \[F(3,40)=14.85, \; p<0.0001\], and an effect of sex \[F(1,40)=34.59, \; p<0.0001\] but there was no interaction \[F(4,40)=1.312, \; p=0.2840\] (see Figure 4.9). There was a significant effect of time block in the combined control data \[F(3,48)=27.06, \; p<0.0001\] as well as a significant effect of sex \[F(1,48)=5.286, \; p=0.0259\] and interaction \[F(3,48)=8.568, \; p=0.0001\].

Two-way ANOVA of path length in the first time block (first 5 minutes) of day 1, showed no significant effect of treatment \[F(1,22)=0.8519, \; p=0.3660\] but there was a significant effect of sex \[F(1,22)=19.18, \; p=0.0002\] with no interaction \[F(1,22)=0.1916, \; p=0.6659\]. Sidak’s multiple comparisons post-test reveals a significant effect of sex in both the alcohol \([p<0.01]\) and control \([p<0.05]\) groups (see Figure 4.11). In time block 1 on day 2 there was no significant effect of treatment \[F(1,22)=0.02983, \; p=0.8645\], a significant effect of sex \[F(1,22)=21.97, \; p=0.0001\] and no interaction
[F(1,22)=0.5586, p=0.4628]. Sidak’s post-test revealed a significant effect of sex in both the alcohol [p<0.01] and control [p<0.05] groups.

Analysis of time block 2 for day 1 revealed similar results, with a significant sex-effect [F(1,22)=7.529, p=0.0118] but no effect of treatment [F(1,22)=0.1142, p=0.7386] or interaction [F(1,22)=0.9797, p=0.3330]. Sidak’s test revealed that there was a sex difference in the alcohol animals [p<0.05] but not the control animals (see Figure 4.11). For time block 2 on day 2 there was a significant effect of sex [F(1,22)=17.75, p=0.0004] but no significant effect of treatment [F(1,22)=0.6675, p=0.4227] or interaction [F(1,22)=1.168, p=0.2915]. Sidak’s test revealed that again sexes were significantly different in the alcohol-exposed group [p<0.01] but not the control group (see Figure 4.11).

For time block 3 of day 1 there was a significant effect of sex [F(1,22)=8.630, p=0.0076] but no significant effect of either treatment [F(1,22)=0.6749, p=0.4202] or interaction [F(1,22)=0.4337, p=0.5170]. Sidak’s multiple comparisons test revealed a significant difference between males and females in the control group [p<0.05] but not the alcohol group (see Figure 4.11). For time block 3 on day 2, there was a significant effect of sex [F(1,22)=14.75, p=0.0009], but not treatment
[F(1,22)=1.933, p=0.1784] or interaction [F(1,22)=0.6611, p=0.4249]. Sidak’s test again revealed an effect of sex in the control group [p<0.01] but not in the alcohol group (see Figure 4.11).

The final time block for day 1 did not show a significant effect of sex [F(1,22)=3.906, p=0.0608], treatment [F(1,22)=0.4190, p=0.5242] or interaction [F(1,22)=0.3247, p=0.5746] (see Figure 4.12). This period in day 2, however, revealed a significant effect of sex [F(1,22)=5.553, p=0.0278] but no significant effect of either treatment [F(1,22)=0.1785, p=0.6768] or interaction [F(1,22)=7.068, p=0.4096]. However, Sidak’s test did not reveal any significant differences between groups on either day.
Figure 4.11  Day 1 and day 2 path length (mean ± SEM) in open field by time blocks, sex and treatment group. M=male, F=female, E=alcohol, C=control. * p<0.05, ** p<0.01
Within treatment groups there was an effect of time block on path length within the open field and so individual time blocks were analysed to determine whether there was a treatment effect that could only be detected within specific time blocks. Each time block was analysed for day 1 and then day 2 in order of time blocks.

Tukey’s multiple comparisons test was used to determine where the differences between time blocks occurred (see Figure 4.12). There were a number of significant differences, both within sex comparing 5-minute time blocks and within a single time block for sex on both days. However, for understanding habituation we report only those time blocks for which path length was significantly different to that of time block 1. On day 1 in control and alcohol-treated males, path-length in time block 2 [control $p<0.0001$; alcohol $p<0.05$], block 3 [control $p<0.0001$; alcohol $p<0.01$] and block 4 [control $p<0.0001$; alcohol $p<0.001$] were less than in block 1. For female alcohol-treated animals, the path-length on day 1 was significantly less in time blocks 2 [$p<0.01$] 3 and 4 [$p<0.0001$] compared to block 1. Female control animal path length was significantly longer in the first time block compared to all other blocks [$p<0.0001$].

In the alcohol-treated animals, there was no difference between male and female animals when compared within a single time block in the first day. In the control animals, females had a significantly greater
path length than males in the first time block on the first day [p<0.01].

On day 2, path length in alcohol-treated males was less for time block 3 than time block 1 [p<0.05]. In control animals, the path length in time block 4 was significantly lower than time block 1 [p<0.05], but there were no significant differences between any of the other time blocks. In female alcohol-treated animals, path length in time blocks 3 and 4 was significantly lower than time block 1 [p<0.001]. Path length was significantly lower in time block 4 compared to time block 1 [p<0.0001] in the female control animals.

Sex differences were investigated again for day 2 as per day 1 within time blocks. Male alcohol-treated animals had a significantly shorter path length than female alcohol-treated animals in time block 1 [p<0.05] and time block 2 [p<0.01]. There were no significant differences in path length between males and female control animals in any single time block for day 2.
The specific location within the open field with which an animal spends time, provides important information on their level of anxiety and exploratory drive. The centre of the maze is more anxiogenic than the outer regions, especially when conducted in bright light (Bouwknecht et al., 2007) conditions such as those used in the current study. Therefore, time spent in the centre of the maze and latency to the centre of the maze was compared between treatment groups and within groups between sexes. As variances

Figure 4.12   Total path length (mean ± SEM) on days 1 and 2 in time blocks of 5-minutes in the open field test. Ethanol=alcohol treated animals

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001
were unequal across treatment groups for the time spent in the centre of the maze, these data were transformed prior to statistical analysis and are presented as a log function of time spent, on the y-axis. The untransformed data are also presented as a scatter graph below (Figure 4.13)

![Figure 4.13 Scatter graphs (mean ± SEM) to demonstrate the unequal variance of duration in the centre of the open field between groups.](image)

Two-way ANOVA revealed there was no effect of treatment on duration spent in the centre of the open field on day 1 [F(1,21)=3.578, p=0.0724]. There was a significant effect of sex [F(1,21)=5.016, p=0.0361], but there was no interaction between treatment and sex [F(1,21)=0.7472, p=0.3791 (see Figure 4.14).
Duration spent in the centre of the open field on day 2, analysed with two-way ANOVA, revealed no significant effect of treatment \([F(1,21)=2.460, \ p=0.1317]\), or of sex \([F(1,21)=3.390, \ p=0.0798]\), and there was no significant interaction \([F(1,21)=3.052, \ p=0.0952]\) (see 4.15). Sidak’s post-test revealed no individual significant differences in either day 1 or day 2.
One-way ANOVA revealed no significant effect of treatment group on the latency to move into the centre of the maze on day 1 \([F(2,23)=0.9212, p=0.6449]\), nor did it detect a difference on day 2 \([F(2,21)=0.6414, p=0.5366]\), however, the treatment groups had unequal variances in the day 2 data (see Figure 4.16).

Figure 4.15 The log y transformation, showing duration spent in the central area of the open field on day 2 of the test (mean ± SEM).
When the control groups were combined to allow sex differences to be investigated, two-way ANOVA did not reveal a significant effect on day 1 data of treatment [F(1,22)=0.5244, p=0.4766], sex [F(1,22)=1.441, p=0.2428] or interaction [F(1,22)=0.5138, p=0.4810]. This was similar to the day 2 data, where there was no significant effect of treatment [F(1,20)=3.571, p=0.0734], and no significant effect of sex [F(1,20)=1.330, p=0.2624] or interaction [F(1,20)=1.603, p=0.2201] (see Figure 4.17).
Figure 4.17: Latency for rats of both sexes and treatment groups (after control animals combined) to move from the sides of the open field into the centre (mean ± SEM) for day 1 (above) and day 2 (below). There were no significant differences detected in these data and no differences were detected by Sidak’s multiple comparisons test.
4.2.2 Novel Object Recognition Test

Sniffing behaviour of familiar objects in rats of both sexes and all treatment groups across the ‘habituation’ trials showed no significant bias for object position \[F(1,200)=0.3439, \ p=0.5583\] (Figure 4.18).

On trial one there was no significant difference in the time spent sniffing between the treatment groups \[F(2,49)=0.1323, \ p=0.8764\]. The time spent sniffing the familiar objects over trials 1 to 3 also showed no significant effect of object \[F(1,150)=0.9392, \ p=0.3340\].
However, trial, within the three habituation trials, did have a significant effect on the time spent sniffing the familiar objects \([F(2,150)=19.71, p<0.0001]\) (Figure 4.19).

The discrimination ratio is a measure of the time spent sniffing the ‘new’ object as a proportion of the total time spent sniffing both objects. A ratio above the threshold level of 0.5 indicates that the animals recognise the new object as novel (Gaskin et al., 2010) and so each group was assessed to determine whether it was performing at a significantly higher level than chance would permit. All treatment groups performed significantly better than chance \([p<0.0001]\) with ethanol animals \((t=5.051, \, df=16)\), intubated controls \((t=13.05, \, df=12)\)
and non-intubated controls (t=6.2, df=10). One-way ANOVA showed there was no significant difference between the treatment groups in discrimination ratio [F(2,23)=1.855, p=0.1792] and so the two control groups were combined to allow investigation of potential sex effects.

Two-way ANOVA revealed a significant effect of treatment on the discrimination ratio, [F(1,21)=8.794, p=0.0074] and a significant effect of sex [F(1, 21)=6.844, p=0.0161] and no interaction [F(1,21)=1.318, p=0.2638]. The Tukey’s post-hoc analysis revealed a significant difference, with alcohol-treated male animals having a significantly lower discrimination ratio than male controls [p<0.05] and female controls [p<0.01] (see Figure 4.20).
Figure 4.2 Figure 4.20 Discrimination ratio for treatment groups shown with dotted line indicating the level at which the new object would be recognized by chance alone = 0.5 (mean ± SEM).

* p<0.05, ** p<0.01
A number of other measures can be used to assess different aspects of the response of animals to a novel object. There was a significant effect of object on latency to first sniff when the novel object was introduced \([F(1,48)=12.18, \ p=0.0010]\) with no effect of treatment \([F(1,48)=0.0726, \ p=0.7887]\) or interaction \([F(1,48)=0.3173, \ p=0.5759]\). Bonferroni’s multiple comparisons test revealed that the ethanol treated animals showed a significantly shorter latency to approach the novel than the familiar object \([p<0.05]\), but control animals did not. This data is shown as a scatter graph to assist in interpreting this finding (see Figure 4.21).
Figure 4.21  The effect of treatment on latency to first sniff of the familiar versus the novel object shown as bar and scatter graph (mean ± SEM). * p<0.05.
When male and female animals were analysed, there was a statistically significant effect of novel object \([F(1,22)=6.193, p=0.0209]\) on the latency to first sniff but no effect of treatment \([F(1,22)=0.08045, p=0.7793]\) or interaction \([F(1,22)=1.341, p=0.2592]\) in the male animals. In the female animals there was no significant effect of object \([F(1,22)=3.817, p=0.0636]\), treatment \([F(1,22)=0.2490, p=0.6228]\) or interaction \([F(1,22)=0.06329, p=0.8037]\) on the latency to first sniff (Figure 4.22). Tukey’s multiple comparisons test revealed no significant differences between the data.
Figure 4.22  Latency to first sniff for male rats (above) and female rats (below).
When the number of sniffing events was investigated, that is the number of times the rat visited the object to sniff there was a significant effect of novel object \( [F(1,48)=42.70, \ p<0.0001] \) but not treatment \( [F(1,48)=0.01263, \ p=0.9110] \) nor was there an interaction \( [F(1,48)=0.4767, \ p=0.4933] \). Tukey’s multiple comparisons test indicated that there was a significant difference in the number of sniffing events for the alcohol animals \( [p<0.01] \) and for the control animals \( [p<0.001] \) (see Figure 4.23).

![Figure 4.23](image)

**Figure 4.23** The number of sniffing events (visits) of the familiar and new object. There was a significant effect of object (mean ± SEM). ** p<0.01, *** p<0.001
4.2.3 Elevated T-Maze

An increase in the length of time spent in the enclosed arm from trial 1 to trial 3 demonstrates acquisition of learned avoidance. There was a significant effect of trial \([F(2,69)=8.345, p=0.0006]\) on learned avoidance but there was no significant effect of treatment \([F(2,69)=1.816, p=0.1703]\) and no interaction \([F(4,69)=0.4266, p=0.7890]\) as shown in Figure 4.24. Bonferroni’s multiple comparisons test revealed no individual differences between treatment groups within trials.

![Avoidance time for each trial and each treatment group in the ‘learned avoidance’ part of the elevated T-maze (mean ± SEM).](image)

**Figure 4.24** Avoidance time for each trial and each treatment group in the ‘learned avoidance’ part of the elevated T-maze (mean ± SEM).
When the control groups were combined there was no effect of treatment \([F(1,72)=2.023, p=0.1592]\) on learned avoidance but there was a significant effect of trial \([F(2,72)=10.91, p<0.0001]\), as shown in Figure 4.25. There was no significant interaction \([F(2,72)=0.7694, p=0.4670]\). Sidak’s post-test did not reveal any significant differences between the treatment groups in each trial of learned avoidance.

Figure 4.25  
Avoidance time for ethanol and combined controls for each trial in the ‘learned avoidance’ part of the elevated T-maze graphed as a column and scatter graph (mean ± SEM).

* \(p<0.05\), *** \(p<0.001\)
Within the alcohol treated animals, trial 1 \([p<0.001]\) and 2 \([p<0.05]\) were shorter than trial 3 indicating that the alcohol animals did show inhibitory avoidance over the three trials but control animals did not.

There was no significant effect of treatment on escape time \([F(2,67)=0.9571, \ p=0.3892]\) or an effect of trial \([F(2,67)=1.569, \ p=0.2157]\) or any significant interaction \([F(4,67)=0.2850, \ p=0.8867]\) (see Figure 4.26).

![Graph showing escape time for each trial and each treatment group](image)

**Figure 4.26** Escape time for each trial and each treatment group in the ‘one-way escape’ part of the elevated T-maze (mean ± SEM).

When the control groups were combined there was no effect of treatment \([F(1,70)=0.5398 \ p=0.4650]\) on the time to escape and there
was also no effect of trial [F(2,70) = 2.107, p = 0.1292], or interaction F(2,72) = 0.7694 p = 0.4670] as shown in Figure 4.27.

Figure 4.27: Escape time for ethanol and combined controls for each trial in the ‘one-way escape’ part of the elevated T-maze graphed as a column and scatter graph (mean ± SEM).
In many studies only one escape trial is used as this reflects innate fear without the potential confound of acclimation to the task. There was however no significant difference between escape trial 1 and later trials.

4.2.4 Morris Water Maze Training

Two-way ANOVA revealed there was significant effect of day \([F(3,92)=60.28, p<0.0001]\) and treatment \([F(2,92)=3.845, p=0.0249]\) on path length. Post-hoc analysis of these data revealed that there were no significant differences between treatment groups within days as seen in Figure 4.28. The control groups were combined and analysis using two-way ANOVA revealed there was a significant effect of day \([F(3,96)=55.14, p<0.0001]\) but no treatment effect \([F(1,96)=0.5979, p=0.8073]\) on path length as seen in Figure 4.29. Bonferroni’s multiple comparisons test revealed no differences between treatment groups on any individual days. There were however individual differences between days within treatment groups when Tukey’s multiple comparisons test was used with significant differences identified between day 1 and day 2, 3 and 4 in both treatment groups \([p<0.0001]\). Differences were also found in the combined control data between day 2 and day 3 \([p<0.01]\) and day 2 and day 4 \([p<0.001]\).
Figure 4.28  Mean path length for each day of training on the reference memory version of the MWM (mean ± SEM). Path length decreased with increasing number of trials with no significant difference between treatment groups.
There was considerable variability between animals in the data when total path length during training was investigated. (See Figure 4.30, below, which plots each trial for each animal each day with each treatment group plotted on a separate graph).
Figure 4.30 The path length for animals over training trials for the reference memory version of the MWM (mean ± SEM). Note that there is considerable variation between animals and between animals of the same treatment group.
Performance on day 1 of training influences all later training gains (Perrot-Sinal et al., 1996; Vorhees and Wiliams, 2006), therefore the average improvement in each day’s path length was analysed by calculating the savings in path length as a percentage of day 1 path length. There was a significant effect of day on the data \([F(2,69)=8.810, p=0.0004]\) however there was no significant effect of treatment on path length reduction \([F(2,69)=0.2722, p=0.7625]\) or a significant interaction \([F(4,69)=0.8295, p=0.5110]\) (see Figure 4.31). Tukey’s multiple comparisons test revealed there were no significant differences between the treatment groups. Further, this test was used to investigate differences in means on individual days within treatment groups and there were no significant differences except within the non-intubated control group between the path length percentage reduction on day 2 compared with day 4 (see Figure 4.31).

Included in Figure 4.31 is a scatter graph to highlight the variability within the data, the control groups were combined to investigate the difference between the alcohol group and a single, non-alcohol-treated control group as per the previous experiments.

If there was less learning in one group on day one this may be detected as a difference in the gain on the second day of training only, and was analysed separately.
Figure 4.31  The percentage improvement against day 1 path length for training on days 2, 3 and 4 of the MWM reference memory version (mean ± SEM). **\ p<0.01
When control groups were combined and the data analysed there was still no significant effect of treatment \([F(1,72)=0.2711, p=0.6042]\) as seen in Figure 4.32, above. There were no significant differences between the treatment groups on individual days, as revealed in Sidak’s multiple comparisons test. There were, however, significant differences in percentages within groups, when savings in path length for day 2 were compared to day 3 and day 4. These differences were only seen in the control animals and not the alcohol-treated animals.

![Figure 4.32](image-url)  
Figure 4.32  The percentage improvement over day 1 path length for training on days 2, 3 and 4 of the MWM reference memory version with control groups combined (mean ± SEM). * \(p<0.05\), *** \(p<0.001\)
Path length in the outer zone of the MWM is a measure of thigmotaxis and is the most common search strategy in the early stages of water maze learning (see section 4.27 Morris Water Maze Search Strategy Analysis). There was a significant effect of day \[ F(3,92)=141.3, \ p<0.0001 \] and also treatment \[ F(2,92)=5.383, \ p=0.0062 \] on thigmotactic behaviour. There was an interaction of treatment with day \[ F(6,92)=2.866, \ p=0.0132 \] as seen in Figure 4.33 below.

![Figure 4.33](image)

**Figure 4.33** Mean path length in the outside zone for each day of training on the reference memory version of the MWM (mean ± SEM). Post testing reveals differences between groups on day 1 average path length. *** p<0.001
Tukey’s multiple comparisons test revealed differences between the non-intubated control group and both the alcohol and intubated control groups on day 1 \[p<0.001\].

As we are investigating training, total path length can vary greatly, depending on if, and when, the animal finds the platform. Therefore, these training measures are more robust when they are converted, on an individual animal basis, to the percentage of the total path length travelled that day (Simon et al., 1994). When these path lengths were converted to a percentage and reanalysed, there was an effect of day \[F(3, 92)=99.60, \ p<0.0001\], but there was no effect of treatment \[F(2, 92)=2.958, \ p=0.0569\] and no interaction \[F(6, 92)=1.258, \ p=0.2845\] as seen in Figure 4.34.

![Graph showing mean percentage of total path length in the outside zone for each day of training on the reference memory version of the MWM (mean ± SEM)](image)

**Figure 4.34** Mean percentage of total path length in the outside zone for each day of training on the reference memory version of the MWM (mean ± SEM)
As there was no significant difference between the treatment groups, the control groups were combined as in previous experiments to increase statistical power. There was no treatment effect \[ F(1,96)=1.126, \ p=0.2912 \] but there was a significant effect of day \[ F(3,96)=97.05, \ p<0.0001 \] but no interaction of day with treatment \[ F(3,96)=1.839, \ p=0.1453 \] on the percentage of path length spent in the outer zone (see Figure 4.35). Tukey’s multiple comparisons test did not reveal any differences between the treatment groups on any single day. Tukey’s post test indicated that for the alcohol exposed animals, the percentage of path length spent in the outer zone was significantly different across the days of: days 1 and 2 \[ p<0.0001 \], days 1 and 3 \[ p<0.0001 \] and days 1 and 4 \[ p<0.0001 \] as seen in Figure 4.35. In the control group percentage of path length spent in the outer zone across the days of training differed between: day 1 and 2 \[ p<0.0001 \], day 1 and 3 \[ p<0.0001 \] and day 1 and 4 \[ p<0.0001 \] and also between day 2 and 3 \[ p<0.01 \] and day 2 and 4 \[ p<0.001 \] indicating additional learning from day 2 to day 3 not seen in the alcohol exposed animals.
The percentage of the path length within the target quadrant (the quadrant containing the platform) would be expected to increase over the training period as animals became aware of the platform location and developed an efficient search strategy. There was a significant effect of day \([F(3,92)=56.35, p<0.0001]\), but no effect of treatment \([F(2,92)=0.3694, p=0.6922]\) and no interaction of day and treatment \([F(6,92)=0.6334, p=0.7032]\). Tukey’s post test revealed no between group differences in percentage in the target quadrant on
any single day of training. As there were no between group differences, the control groups were combined as above.

With control groups combined, there were no statistical differences between treatment groups \( [F(1,96)=0.01329, \ p=0.9085] \) but there was a significant effect of day on the data \( [F(3,96)=58.70, \ p<0.0001] \) as revealed by two-way ANOVA. There was no significant interaction between day and treatment \( [F(3,96)=1.221, \ p=0.3065] \). Sidak’s post test revealed no significant differences between treatment groups on any single day (see Figure 4.36).

**Figure 4.36**  Mean percentage of total path length in the target quadrant for each day of training in the reference memory version of the MWM (mean ± SEM). * p<0.05, *** p<0.001, **** p<0.0001
Tukey’s post-hoc test was used to compare the multiple days within treatment groups and revealed significant differences. In the alcohol-treated animals, there were significant differences between: day 1 and day 2 \([p<0.0001]\), day 1 and day 3 \([p<0.0001]\), day 1 and day 4 \([p<0.0001]\), and day 2 and day 4 \([p<0.05]\). In the combined control group, there were significant differences between: day 1 and day 2 \([p<0.001]\), day 1 and day 3 \([p<0.0001]\), day 1 and day 4 \([p<0.0001]\), day 2 and day 3 \([p<0.05]\), and day 2 and day 4 \([p<0.0001]\) as seen above, in Figure 4.36. This different pattern of the percentage of path length spent in the target quadrant with training day reflects subtle changes in training performance.

Latency to platform can also be used as a measure of performance, but there is a potential confound in this measure. If the rat pauses in its swim search to look around and assess visual cues, the latency increases but path length would not. However this analysis was included as any difference between the measures would be of interest in elucidating the strategy used. There was a significant effect of day \([F(3,92)=45.53, p<0.0001]\), but no effect of treatment \([F(2,92)=2.788, p=0.0668]\) and no interaction of day and treatment \([F(6,92)=1.029, p=0.4118]\) (see Figure 4.37). Tukey’s multiple comparisons test revealed no statistical differences between
treatment groups on any single day of training. As there was no significant difference between the treatment groups, the control groups were combined to increase power for subsequent analysis. With the control groups combined, there was no significant effect of treatment on the data \([F(1,96)=0.5452, p=0.4621]\) but there was a significant effect of day of training \([F(3,96)=43.38, p<0.0001]\). There was no interaction between treatment and day \([F(3,96)=1.573, p=0.2008]\). Sidak’s multiple comparisons test did not reveal any between group differences within any single day of training. There were however, significant differences in latencies within treatment groups between days of training. In the alcohol-treated animals there was a significant difference between day 1 and days 2, 3 and 4 \([p<0.0001]\). In the combined control animals, there was a significant difference between day 1 and days 2, 3, 4 \([p<0.0001]\). There was also a significant difference between day 2 and day 3 \([p<0.05]\) and day 2 and day 4 \([p<0.01]\), note there was no significant difference between these days in the alcohol–treated group.
Swimming speed was calculated for each animal on each trial from the time taken (seconds) to travel the path length. There was a significant effect of day \([F(3,92)=12.70, \ p<0.0001]\), but no effect of treatment \([F(2,92)=3.086, \ p=0.0504]\) and no interaction of day and treatment \([F(6,92)=0.8302, \ p=0.5495]\). Post hoc multiple comparison testing found that in the alcohol-treated group swimming speed was
slower on day 4 than day 1 \([p<0.05]\). For the non-intubated control animals, speed was also slower on day 4 than day 1 \([p<0.01]\) and in the intubated control group, swimming speed was slower on both day 3 and day 4 compared to day 1 \([p<0.01]\) (see Figure 4.38).

![Swimming speed across all days of training in the reference memory version of the MWM for all groups (mean ± SEM).](image)

* \(p<0.05\), ** \(p<0.01\)

Data for the two control groups were combined due to the lack of a treatment effect and it was considered that a larger number of control animals might elucidate how swimming speed relates to training and search strategy. There was a significant effect of day
[F(3,96)=12.18, p<0.0001], and an effect of treatment [F(1,96)=4.346, p=0.0397] but there was no interaction of day and treatment [F(3,96)=0.4338, p=0.7293]. Tukey’s multiple comparisons test found that there was no effect of treatment on any of the days but there were different patterns in the effect day had on swimming speed in the alcohol-treated animals compared with the controls. The swimming speed of the alcohol-treated animals was significantly different between day 1 and day 4 [p<0.05], and day 2 and day 4 [p<0.05]. The swimming speed of the combined control animals was also different between day 1 and day 4 [p<0.001] and day 2 and day 4 [p<0.01] but the control animals were also swimming more slowly by day 3 compared to day 1 (p<0.01) (see Figure 4.39).
4.2.5 Morris Water Maze - Reference Memory Probe Trial

Several measures of performance on the probe trial were analysed, as there is conflicting opinion as to the most reliable estimator and it has been shown that the detection of group differences differs between commonly used parameters (Maei, et al., 2009). As the current study detected minimal differences in performance in the training measures of the MWM reference memory version we chose to assess probe performance in a variety of...
of ways. There was no significant effect of treatment on the percentage of trial duration (60 seconds) in each quadrant (previous platform location was in the South West quadrant) \([F(2,92)=0.007754, p=0.9923]\) (see Figure 4.40) or on the path length in each quadrant \([F(2,92)=0.7800, p=0.4614]\) (see Figure 4.41). However there was an effect of quadrant on both swim duration \([F(3,92)=48.41, p<0.0001]\) and path length \([F(3,92)=44.34, p<0.0001]\).

![Figure 4.40](image)

**Figure 4.40** Percentage of the total probe trial time of the reference memory version of the MWM spent in each of the four quadrants for each of the three treatment groups (mean ± SEM). Lone asterisks denote difference to SW quadrant (target). * \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\), **** \(p<0.0001\)
Tukey’s post-test revealed that within each quadrant there were no significant differences between groups for either the path length or the duration of the swim period (see Figures 4.40 and 4.41). Tukey’s post-testing however did reveal within group differences between quadrants. When the Tukey’s post-test was applied to the percentage
duration in each quadrant, alcohol-treated animals had a significantly different target quadrant duration percentage in the SW quadrant (target platform) to all other quadrants (SE [p<0.0001], NW [p<0.01], NE [p<0.0001]) and the NW and SE were also significantly different (p<0.001) (see Figure 4.40). The two control groups showed a significantly different percentage of the total swim time duration in the SW target quadrant compared to the SE (IC [p<0.0001], NIC [p<0.0001]) and the NE (IC [p<0.001], NIC [p<0.0001]) but not NW (see Figure 4.37). They also showed a significant difference between the percentage duration in SE versus NW (IC [p<0.01], NIC [p<0.001]) and the NIC animals in NW compared to NE (p<0.01) (see Figure 4.40).

The path length travelled was significantly different to the SW quadrant, for ethanol-exposed animals, in the SE [p<0.0001], the NW [p<0.05] and the NE [p<0.0001] quadrants. There were also additional significant differences between SE and NW, and NW and NE quadrants (see Figure 4.41). In intubated control animals the SW quadrant was significantly different to the SE [p<0.0001] and the NE [p<0.0001] quadrants but was not significantly different to the NW quadrant [p>0.05]. Additional significant differences in path length travelled occurred in between SE and NW and NW and NE quadrants (see Figure 4.41 for details). The non-intubated animals’
data showed a significant difference between the SW quadrant and both the SE \( p<0.0001 \) and the NE \( p<0.001 \) but again, there was no difference to the NW quadrant. There was a significant difference between the SE and NW, and the NW and NE quadrants (see Fig 4.41).

The percentage of the total path length that occurred in the platform zone, namely within a 10cm circle centred on the location of the training platform is shown in Figure 4.42. A one-way ANOVA revealed there was no significant effect of treatment \( F(2,23)=1.489, \ p=0.2465 \).

![Figure 4.42](image)

Figure 4.42  Percentage of the path length during the 60-second probe trial of the reference memory version of the MWM that occurred within the platform zone (mean ± SEM).
The number of probe trial crosses over the area that was occupied by the platform during the training trials was not significantly different between treatment groups \([F(2,23)=1.825, \ p=0.1838]\) (see Figure 4.43). As this measure was not different between treatment groups, the two control groups were combined to increase statistical power (see Figure 4.44).

![Figure 4.43](image)  
Mean number of times the previous location of the platform was crossed for each of the three treatment groups during the 60 second probe trial of the reference memory version of the MWM (mean ± SEM).
An unpaired t-test performed on the number of platform crosses with two treatment groups did not detect a significant difference between the groups \[t(24)=1.796, p=0.0851\].

A further test was used to investigate the animals’ behaviour over the 60-second probe trial, which was to record and analyse the animals’ proximity to the platform (Gallagher et al., 1993). To do this,
the TopScan software (TopScan 2.0, Cleversys Inc™, USA) was set up to record the animal’s distance from the platform every second for each second of the trial and average these distances to give each animal a number for comparison. One-way ANOVA revealed no significant differences between treatment groups in this test [F(2,23)=0.4631, p=0.6351], however it was detected that the standard deviations of the treatment groups were uneven, with more variation in the intubated control group (see Figure 4.45).

Figure 4.45  Mean proximity to platform for the three treatment groups over the 60 seconds of the probe trial of the reference memory version of the MWM (mean ±
Behaviour of the animal in the first five seconds of the probe trial may be more instructive of performance, as failure to find the platform immediately in its learned location may result in thigmotaxis, random searching or other time occupying behaviours. Whishaw’s corridor was used to compare the actual path length in the first 5 seconds to an ideal corridor between start location and platform (Arqué et al., 2008). There was no effect of treatment on path length in Whishaw’s corridor \[ F(2,23)=0.1407, \ p=0.8695 \] (see Figure 4.46). Proximity to the platform during the first 5 seconds of the probe trial was also not significantly affected by treatment \[ F(2,23)=0.4965, \ p=0.6150 \] (see Figure 4.47).
Figure 4.46  The percentage of the total path length that occurred in Whishaw’s corridor during the first five seconds of the probe trial of the reference memory version of the MWM (mean ± SEM).
This study included male and female animals within each group but numbers were not sufficient to do statistical analysis of sex effects across all treatment group. As no differences were detected between the intubated and non-intubated control groups they were combined to allow sex analyses within treatment group with at least n=6 per group.

Figure 4.47  Mean proximity to platform for the three treatment groups over the first five seconds of the probe trial of the reference memory version of the MWM (mean ± SEM)
Two-way ANOVA revealed no significant effect of sex \( [F(1,22)=0.0007842, p=0.9779] \) or treatment \( [F(1,22)=3.953, p=0.0594] \) on the number of times animals crossed over the site of the platform location. However there was a significant interaction between sex and treatment \( [F(1,22)=7.379, p=0.0126] \) and Sidak’s multiple comparisons test revealed that alcohol-treated male rats completed significantly less platform crossings than control males \( [p<0.01] \) (see Figure 4.48). The performance of the control males did not differ from either alcohol-treated or control females. The alcohol-treated males would have ‘found’ the platform on average only once in the 60-second trial while control males would have ‘found’ the platform on average three times.
As a measure of thigmotaxis, duration in the outside 10 cm of the maze was analysed (see Figure 4.49). Duration was used rather than path length as an animal will often stay in one spot and attempt to climb the side of a tank in an attempt to escape the water, or merely stop for a period of time. There was a significant effect of both sex \([F(1,22)=10.13, p=0.0043]\) and treatment \([F(1,22)=4.385, p=0.0480]\) on thigmotactic behaviour but there was no significant interaction.
[F(1,22)=1.773, p=0.1966]. Post-hoc analysis using Sidak’s test revealed a significant difference between alcohol and control groups for female animals [p<0.05] but no significant difference for males. Within the alcohol treated animals there was also significant difference between the male and female animals [p<0.05] but this was not present in the control group.

Figure 4.49  Duration in the outside of the water maze, an indicator of thigmotactic behaviour, during the probe trial of the reference memory version of the MWM (mean ± SEM). * p<0.05
Latency to the platform on the first approach is shown in Figure 4.50. This measure suggests how quickly the animals would have found the platform if it was in its original position. There was no significant effect of sex \([F(1,22)=3.409, \ p=0.0783]\), treatment \([F(1,22)=0.06484, \ p=0.9365]\) or any interaction \([F(1,22)=1.074, \ p=0.3114]\). The Sidak’s multiple comparison post-test did not reveal any significant individual differences.

![Graph showing latency to platform](image)

Figure 4.50 The effect of treatment and sex on latency to reach the previous platform location for the first time in the probe trial of the reference memory version of the MWM (mean ± SEM). There was no significant effect of treatment or sex.
4.2.6 Morris Water Maze Reversal Training

The average distance travelled over four trials on the first and second day of reversal testing is shown in Figure 4.51. Two-way ANOVA revealed no significant effect of treatment [F(2,45)=1.184, p=0.3155] or interaction [F(2,45)=0.3434, P=0.7112] on the mean distance travelled. There was also no significant effect of day [F(1,45)=3.452, P=0.0697]. Sidak’s post-test also revealed no between group differences on any single day or within group differences across days.

Figure 4.51 Mean path length on both days of reversal training on the MWM (mean ± SEM). There was no significant difference of treatment or day or interaction.
The number of times the animals crossed the previous platform was also investigated on the reversal testing days. This parameter gives a measure of the animals’ searching in the old platform location to investigate the animals’ ability to stop searching in the old location when the platform had moved. There was no significant effect of treatment \([F(2,45)=1.901, p=0.1613]\), but there was an effect of day on the number of platform zone crosses \([F(1,45)=10.07, p=0.0027]\) and no interaction \([F(2,45)=1.333, p=0.2740]\). Tukey’s post testing revealed a significant difference between NIC and IC animals on day 1 \(p<0.05\) and in the NIC treatment group there was a significant difference between crosses on day 1 and day 2 \(p<0.05\) (see Figure 4.52).

It is possible that differences may occur in performance on the first trial of each day and so individual trials were analysed with respect to treatment and day and are shown below in Figure 4.53. There was a significant effect of trial on the data \([F(7,180)=6.559, p<0.0001]\), but there was no significant effect of treatment \([F(2,180)=1.863, p=0.1581]\) and no interaction of trial and treatment \([F(14,180)=0.8821, p=0.5793]\). Tukey’s multiple comparisons test revealed no differences between any treatment groups on any trials.
However in both the IC and NIC groups there were significant differences between trial 1 and various other trials (see Figure 4.53 for details).

As there was no statistically significant difference between the control groups, these were combined to assess whether this elucidated any differences in performance between alcohol-treated animals and controls. There was a significant effect of trial on the data \([F(7,188)=6.204, p<0.0001]\), but there was no significant effect of
treatment \[F(1,188)=0.1411, p=0.7076\] and no interaction of trial and treatment \[F(7,188)=0.9362, p=0.4798\]. Tukey’s multiple comparisons test revealed no differences between ethanol-exposed and control animals on any trials. However the control group animals showed a significantly shorter path-length on each trial compared to trial one (see Figure 4.54 for details).

Figure 4.53  The path length for each trial across the two days of reversal training on the MWM (mean ± SEM). There was no significant effect of treatment on any trial. (effect of trial, compared to Trial 1: IC *; NIC ∞). * p<0.05, ** p<0.01, *** p<0.001; ∞ p<0.05
There is no ‘best’ strategy, for an animal to use when searching for the platform in the reference memory version of the MWM, however there is a hierarchy from thigmotaxis to random searching to spatial search strategies. A set of search strategies was defined (Graziano et al., 2003) and colour coding was used in pie graphs to denote the specific search strategies. For each trial, each strategy was converted
to the proportion of the trial for which it was used and the predominant search strategy was assigned as the strategy used for that trial. This data was used to assess whether there was an effect of treatment on the search strategy used on each day of training and importantly to assess how the different treatment groups adapted their search strategy over time, a within group analysis. Data was assessed for all treatment groups (see Figure 4.55) and with control groups combined.

The proportion of animals using each strategy is shown for all treatment groups in Figure 4.55.
Chapter Four: Experiment Three

Day 1
- Ethanol
- Intubated Control
- Non-intubated Control

Day 2
- Ethanol
- Intubated Control
- Non-intubated Control

Day 3
- Ethanol
- Intubated Control
- Non-intubated Control

Day 4
- Ethanol
- Intubated Control
- Non-intubated Control

Legend:
- thigmotaxis
- circling
- random searching
- self-orienting
- approaching target
- scanning
- direct finding
Figure 4.55  This figure (opposite page) shows the relative use of each search strategy (mean over four trials) on each day of training on the reference memory version of the MWM. The data represents the proportion of daily searching that used each of the defined strategies to show how the pattern of searching changed during training for all treatment groups.

Within the ethanol-treated animals there was an effect of strategy [F(6,84)=3.522, p=0.0037] but no effect of day [F(3,84)=0.0, p>0.0999] but there was an interaction of day and strategy [F(18,84)=2.932, p=0.0005]. Tukey’s post hoc test found the majority of significant effects of strategy occurred on day 1 of training with thigmotactic behaviour being the most commonly strategy compared to circling [p<0.001], random searching [p<0.05], scanning [p<0.01], self-orienting (NS), approaching target [p<0.01] and direct finding [p<0.01]. Other significant strategy differences did occur but all were in comparison to thigmotactic behaviour on day 1 and other behaviours on subsequent days. Thigmotactic behaviour on day 1 was also significantly greater than on day 2 [p<0.01], day 3 [p<0.001] and on day 4 [p<0.001]. A similar pattern in the change in search strategy with day was found in the IC group where there was a significant effect of strategy [F(6,84)=2.324, p=0.0399] but not day [F(3,84)=0.0, p>0.0999], and a significant interaction [F(18,84)=2.624,
Tukey’s post-test found that all significant differences occurred between thigmotactic behaviour on day 1. Thigmotaxis on day 3 \( [p<0.01] \) and on day 4 \( [p<0.01] \) was less common with thigmotactic behaviour also significantly more common than random searching \( [p<0.01] \) and direct finding \( [p<0.05] \) on day 1.

Search strategy in the NIC animals showed a significant effect of strategy \( [F(6,83)=6.070, \ p<0.0001] \) but not day \( [F(3,83)=0.009731, \ p=0.9987] \), and a significant interaction \( [F(18,83)=4.185, \ p<0.0001] \). Tukey’s post-test found that thigmotactic behaviour on day 1 was a significantly more common strategy than on day 2 \( [p<0.01] \), day 3 \( [p<0.0001] \) and day 4 \( [p<0.0001] \). On day 1 thigmotaxis was more commonly used than random searching \( [p<0.001] \), scanning \( [p<0.0001] \), self orienting \( [p<0.01] \), approaching the target \( [p<0.0001] \), direct finding \( [p<0.001] \) but not circling.

The two control groups were combined as there was no effect of treatment \( [F(2.12)=0.0025, \ p=0.9975] \) on any of the search strategies and it was considered that larger numbers in the control group might elucidate further differences in strategy selection over training days. Within the combined control there was a significant effect of strategy \( [F(6,84)=4.825, \ p<0.0003] \) but not day \( [F(3,84)=0.03376, \ p=0.9916] \), and a significant interaction \( [F(18,84)=4.590, \ p<0.0001] \). Tukey’s post hoc testing was particularly interesting when the more
random search patterns were compared with thigmotactic behaviour being more prevalent on day 1 than day 2 \([p<0.001]\), day 3 \([p<0.0001]\), and day 4 \([p<0.0001]\). Thigmotactic behaviour on day 1 was also significantly more common than circling on day 1 \([p<0.01]\) and random searching \([p<0.001]\) as well as the intermediate strategy of scanning \([p<0.001]\) and the focussed strategies of self orienting \([p<0.01]\), approaching the target \([p<0.001]\) and direct find \([p<0.001]\).

### 4.3 Discussion

The overall aim of this study was to investigate the effects of binge alcohol exposure on the brain and behaviour of the developing adolescent. As the behavioural tests described in this chapter were performed a considerable time after experimental treatment with alcohol, we were assessing changes that could be present in a mature adult, i.e. the long-term effects of adolescent intermittent binge alcohol exposure on behaviour. This has allowed us to speculate on the behavioural deficits seen in adults that could be attributed to binge-type adolescent alcohol exposure. To allow this extrapolation to the human, we have used an animal model that we consider to be a more reliable model of human adolescent drinking than those used to obtain much of the previously published data (Crews et al., 2000;
Pascual et al., 2007; Nixon et al., 2008). This however does introduce the possibility that the effects, or lack thereof, in the current study, may in fact be the result of the paradigm used not being directly comparable to previously used models whose use populates the scientific literature; the issue of model relevance will be discussed in the final section of this thesis. However, it also highlights that adolescent alcohol exposure, similar to foetal alcohol exposure (Bonthius and West, 1991), will have a range of outcomes dependent on key factors such as the dose of alcohol, blood ethanol concentration and frequency of exposure.

Overall, the animals that experienced episodic binge alcohol exposure throughout adolescence showed only subtle differences in performance on a range of behavioural tasks compared to the control animals. These differences were either very specific to the task or were gender dependent; this specificity has been noted previously, following alcohol exposure in adolescent animals (Pascual et al., 2007; Medina et al. 2008; Forbes 2013). Each specific task used in the current study was selected to assess the functionality of a particular brain structure/pathway or as a direct comparison to pre-existing scientific literature. For this reason, specific tests are discussed individually below.
The first in the battery of behavioural tests was the widely used open field test. Rats in their natural environment are highly predated and thus display behaviours associated with an innate, constant state of risk-management (Whishaw and Kolb, 2005). The open field test is seen most simplistically as a test that puts the rats’ risk management behaviour and innate exploration behaviour into conflict and is often called a test of an animal’s ‘emotionality’ in an environment that is usually vastly different to any other environment it has encountered (Denenberg, 1969). It is agreed, however, that the behaviours exhibited in this test are complex and the most commonly measured aspect of exploration, path length, is not purely indicative of emotionality (Archer, 1973; Johnston & File, 1999). In the current experiment, the large, brightly-lit, opaque white, high-walled, and empty, open field apparatus is very different and probably more threatening than the animal’s home cage eliciting a complex emotional response.

Experimental treatment can influence an animal with respect to commonly measured behaviours in open field tests (Denenberg, 1969; Elliott & Grunberg, 2005; Sáenz, et al., 2006). A literature search did not reveal any data relating to open field behaviour in adulthood.
after adolescent alcohol exposure, as in the current study. However
previous studies involving alcohol have shown that this drug does
not acutely affect performance on this behavioural test when given at
non-sedative doses (Rex et al., 1998) and that during withdrawal
from alcohol, adolescent and adult animals are similarly hypoactive
in the open field test, without increased anxiety (Slawecki & Roth,
2004). A literature search found no studies that investigated open
field behaviour after an extended period such as that used in the
current study.

In this study, the 20-minute open field test was analysed as a
whole and as four, five-minute blocks, similar to previous work
(Smith et al., 1999). Adolescent alcohol exposure did not affect
distance travelled for either the male or female animals but there
were interesting sex effects in both treatment groups. All females
had higher ambulation in block 1 but this occurred only in the
alcohol group in block 2 versus the control group in block 3. This
may be due to small differences that are sex and treatment
dependent but is probably not of any behavioural importance as this
effect was not present in the last block. It is interesting to note that
the long-test methodology, which allows the investigation of
individual time blocks, does provide additional information. It has
informed us that sex-based differences occur, as would be seen in the more commonly used, shorter tests (Barrett and Ray, 1970; Blizard et al., 1975; Mechan et al., 2002; Bouwnecht et al., 2007) however, there is a limited temporal nature of this difference that is only observed in the longer test. Under some experimental circumstances this could provide essential information, as when hyperactivity is an outcome of treatment (Bond and Di Giusto, 1976).

The sex differences observed, with females exploring more than their male counterparts, irrespective of treatment is consistent with the literature, as female animals are more active in the open field (Barrett and Ray, 1970; Blizard et al., 1975; Johnston & File, 1991; Elliott & Grunberg, 2005). The reasons for this are not well understood but it is generally suggested that male rodents are more reactive to emotionality than females (interestingly, this is a reversal of what is seen in humans (Gray, 1971)) and that emotionality makes up at least part of the behaviour in the open field test (Archer, 1975). It has been suggested that in response to a fear-inducing stimulus, females display a greater ambulatory ‘escape’ response than males, which tend to become immobile and defecate (Archer, 1975) and that the initial period in the open field constitutes such a stimulus (Welker, 1957). This would explain our observations in the current
experiment, and the lack of significant sex difference in locomotion after 15 minutes. Familiarity with the apparatus did not have a significant influence on day 2 path length. However, fewer significant differences between time blocks within treatment groups on the second day of open field testing does suggest some effect of familiarity as there appears to be a more consistent amount of ambulation across all time blocks.

There was no significant effect of treatment on the time spent in the centre of the open field, which further supports the idea that alcohol treatment did not have an effect on emotionality in the open field. While ambulation can be ambiguous, as discussed above, spending time in the sides of the open field versus the centre can be seen as being emotionality related (Archer, 1974; Bouwknecht et al., 2007). There was a significant effect of sex, with male animals spending a significantly greater amount of time in the centre of the open field. Previous studies have suggested that males display less emotionality, entering the centre of the open field more than females, this supported by lower latencies to enter this zone (Archer, 1975).

The latency to enter the centre of the arena was investigated in the current study; however, there was not a significant effect of
treatment, sex or any interaction. Latency to enter the centre of the maze can be very highly variable, as animals that do not enter this zone will record a score that is equal with the total period spent in the open field (Archer, 1974). However, the second day of open field testing gave more consistent results, as almost all animals entered the centre before the end of the experiment. With a greater number of rats, this experiment may yield interesting data relating to the effect of adolescent alcohol exposure on rats’ anxiety-related behaviour in the open field test, as will be justified below.

The scatter graph of duration in the centre of the open field (Figure 4.13) shows that there appears to be a wider spread of data points in males compared with females with respect to the duration in the centre of the open field in day one. One would expect variation to be greater in females as previous studies have demonstrated that the stage of the oestrus cycle can have a significant effect on these behaviours in the open field (Archer, 1975). As non-ovarectomised female rats were used in the current study, we would expect to see greater variation in this group. Female animals’ show a higher degree of ambulation, as mentioned previously, which has been attributed to a tendency towards active-escape behaviour (Archer, 1973). Escape behaviour would preclude
the centre of the open field, as there is no escape from this area, and animals would stay in the periphery of the arena, which would explain the above data where females have consistent, low durations in the centre of the maze compared to males, which are highly variable.

The novel object test was included in the current study as it is a test that can be used to elucidate differences in ‘pure’ working memory (Ennaceur & Delacour, 1988; Mumby et al., 2002), that is, without any prior rule-learning or subsequent reward or punishment. As working memory is a complex phenomenon, involving networks incorporating multiple brain regions including the prefrontal cortex (Carpenter et al., 2000) and in some tasks, cingulate cortex (Burgess et al., 2001), it is an ideal behaviour to test after the brain has been exposed to alcohol during development. Both the prefrontal cortex and cingulate cortex have been implicated as brain regions that could be vulnerable to the effects of alcohol on adolescent development (see Section 1.2.1-1.3.1). As alcohol acts diffusely across the entire brain the novel object recognition test is an important assessment tool as it is a task that animals can still perform with damage to the hippocampus (Mumby et al., 2002).
In the current study, there was significantly reduced performance in the male animals that had been exposed to alcohol during adolescence, as evidenced by a significantly lower discrimination ratio in this group compared to both the male and female controls. Other studies in the published literature reveal that female rats perform better than males in the novel object recognition task (Sutcliffe, 2007; Grayson, 2007), and this is likely due to the presence of excitatory oestrogen receptors on temporal and frontal brain regions involved in memory in the female rat (Shughrue et al., 1997; Shughrue and Merchenthaler, 2000). However, in the current study, there was no significant difference between male and female control animals with respect to their discrimination ratios on the novel object task. This is most likely due to experimental design, as differences in familiarization trials and inter-trial interval (Ennaceur and Delacour 1988) and/or strain variations can all impact on behavioural performance (Archer, 1974). The design of the experiment in the current study could be considered ‘harder’ than normal, as there were relatively small differences between objects and a large inter-trial interval. If the experiment had been made easier it is unlikely the deficit in alcohol-treated male animals would have been discovered.
Another important part of the experimental design used here was ensuring that the animals did not show a bias toward a certain object during habituation or a certain area of the arena. The results show no significant difference between the amount of sniffing during habituation with respect to the position of the object or of the object itself. Therefore the findings can be interpreted as being solely due to differences in perception of the objects.

When the latency to sniff the novel object was analysed, there were no significant differences between the latencies to sniff the new versus old object in the control group. Alcohol-treated animals had a significantly lower latency to sniff the new object, which was unexpected. However, the scatter graph (Figure 4.21) reveals that the individual animals showed very similar responses across treatment groups for the novel object but the data were highly variable with respect to their interest in the familiar object. This suggested that some animals might have spent a much longer time exploring the novel object leaving little time to approach the familiar.

In line with the discrimination ratio data, the combining of control groups allowed the investigation of a sex effect on latency. The ANOVA showed a significant effect of the novelty of the object on
the latency of male but not female animals but post-hoc testing did not show specific group differences in the male group. In the data (see Figure 4.22) the SEM for the females was very large for the ‘old’ object latency measures indicating that animals had a highly variable interest in the old object as may be expected. However this does suggest that all males were more likely to recognize the object as novel and approach it in contrast to the females.

Animals of both treatment groups made a greater number of sniffing/exploration events on the new object than the old object. These data, similar to the time spent sniffing, take into account behaviour during the entire five-minute trial. Interestingly, while both alcohol and control animals sniffed the novel object on a significantly greater number of occasions, compared to the old object, the control data had a higher degree of significance (see Figure 4.23). This difference cannot easily be detected looking at the graph so is unlikely to be a significant difference between treatment groups.

Working memory is used to identify novel objects and it is very complex, involving frontal and temporal cortex structures including, as stated above, the prefrontal cortex and the cingulate cortex. The
cingulate cortex is involved in inhibitory control (Velanova et al., 2008), while regions of the prefrontal cortex are likely to be essential for episodic memory (Lepage et al., 1999), which is memory of ‘episodes.’ It is, therefore, unsurprising that these two regions may both be involved in working memory, however their contribution is not well understood and not universally supported (Ennaceur et al., 1997). Neuroimaging studies have revealed that there are at least three separate prefrontal cortex to ventral temporal cortex networks which are involved in working memory concerning different types of cues (Ishai et al., 1999) and that there is a certain degree of overlap in their function (Ishai et al., 1999; Carpenter et al., 2000) which could represent a redundancy of function (Carpenter et al., 2000). A redundancy of function in these networks may explain why previous research has not detected a functional loss resulting from damage to the medial prefrontal cortex (Ennaceur et al., 1997). The window for prefrontal cortex development falls within adolescence and thus our alcohol treatment period, as does development of the cingulate cortex (Velanova et al., 2008). Therefore any behavioural task that involves use of these two brain regions is of great interest in regards to this topic of long-term adolescent alcohol exposure effects on brain and behaviour.
In support of our hypothesis, studies using a cannabinoid receptor agonist revealed that cannabinoid exposure for 21 days from PN30 throughout adolescence caused working memory deficits in adult rats, as measured by a novel object recognition test (O’Shea et al., 2004; 2006). A deficit was not detected in an adult-exposed group, suggesting that this effect was due to some adolescent-specific vulnerability. There has been no equivalent work using alcohol, however chronic alcohol use in adult mice (Beracochea et al., 1992) and adult rats (Irle and Markowitsch, 1983) does impair working memory in the delayed alternation task, which is a spatial working memory task. Therefore this study is the first evidence that adolescent alcohol exposure can negatively affect adult novel object recognition performance.

The elevated T-maze was used as part of the battery of tests in this study as it is a reliable test for investigating two distinct types of fear or anxiety, conditioned and unconditioned (Viana et al., 1994). The conditioned fear response is represented by the rats’ exit of the closed arm, whereas unconditioned fear is represented by the rats’ exit of the open arm. Normal performance by control animals would show no habituation to the unconditioned fear of the open arm, with short latencies to move into the closed part of the maze (Viana et al.,
In comparison, repeated trials of exit from the closed arm would show an increasing latency as the open arm is anxiogenic and animals will avoid it by staying in the closed arm. As there was no published literature investigating the behaviour of adult rats, that have experienced an adolescent alcohol regimen, on the elevated T-maze, the current study will be compared to data involving other behavioural tests of anxiety.

In the current study, there was no effect of treatment but there was a significant effect of trial on learned avoidance. Although the latencies to leave the closed arm appeared to increase with subsequent trials, post hoc tests did not reveal significant differences between any data sets. This result was present when three treatment groups were analysed and was maintained when the control groups were combined. The scatter graph, Figure 4.25, shows that avoidance latency varied markedly between animals, particularly on trial 3, probably contributing to the lack of significant differences between data sets.

Although the elevated T-maze was developed to differentiate types of fear (Viana et al., 1994), the majority of studies continue to use the plus maze. During withdrawal from chronic alcohol treatment, adult animals showed less entries into open arms than
controls when tested on the elevated plus maze (Cagetti et al., 2003; Cagetti et al., 2004). However, this result was likely due to alcohol withdrawal behaviours, as similar testing much later following withdrawal did not show this effect in either adolescent or adult animals (White et al., 2000; Schulteis et al., 2008).

The one-way escape data in this study, showed latency values greater than in other studies (normally only the time it takes the rat to move off the arm); however, as expected, latency remained constant between trials. Latency values recorded here were higher than in other studies as a 30-minute habituation phase to the open arm was used on the day prior to testing. This was performed to increase escape latencies, and thus ensure that the one-way escape latency was not so rapid as to prohibit detection of between group differences. As there were no significant differences between treatment groups, the current study suggests that adolescent alcohol exposure does not result in change in the conditioned or unconditioned fear responses in adulthood. Previous studies have also found no effect of adolescent alcohol exposure on adult fear behaviour (White et al., 2000; Schulteis et al., 2008).
In the current study, rats’ acquisition of reference memory was measured by performance on training trials of this version of the Morris water maze. Surprisingly, there was no significant effect of treatment on path length for the four days, four trials per day, of training with a fixed submerged platform. This did not support our hypothesis, as it was expected that the alcohol exposure regimen that was used during adolescence would result in long-lasting deficiencies in spatial memory acquisition. Alcohol has been shown to cause acute deficits in acquisition of reference memory of adolescent rats (Markweise et al., 1998; Sircar et al., 2009) however; effects of alcohol on spatial learning are not long lasting (Silvers et al., 2003; Cagetti et al., 2004; Schulteis et al., 2008) with alcohol-treated animals performing similarly to controls after several days of abstinence. In the current study, when control groups were combined, there was still no effect of treatment.

Path lengths recorded in the current study are comparable with previous studies (Chesler and Juraska, 2000) using Long-Evans rats, however acquisition in the current experiment took longer as training comprised of 16 trials over 4 days in contrast to one day. Spatial memory performance is also affected by age (Gage et al., 1984; Rapp et al., 1987) and strain, however Long-Evans are less
affected by advanced age than some other rat strains, even at considerably more advanced age than in the current study (Lindner and Schallert, 1988). Rats did reach a level of performance consistent with the literature after several days of training. The use of older rats in the current study was intentional as it allowed us to investigate whether spatial memory deficits caused by adolescent alcohol exposure were present in later life, either as a result of persistence of an adolescent alcohol-induced effect or as an alcohol-induced aging effect. This study however was not designed to differentiate between these possible scenarios.

Post-hoc analysis revealed that there were significant differences in both groups between day one path length and all other days, because path lengths were significantly reduced as platform location was learned. Therefore to investigate subtle differences in acquisition rate, each day of training was analysed as a percentage improvement of path length compared to day one. In the alcohol-treated group there was no significant difference between path lengths on training days as a percentage improvement of day one, but the control animals showed significantly more improvement on day two compared to day three and day four. Although subtle, this is important as it shows that although learning occurs in the alcohol-
exposed adolescents the rate of learning is different and has a slower improvement. This may impact on aspects of life experience.

There was a significant effect of day and treatment on thigmotaxis, and post-hoc analysis revealed significant differences between groups for path length in the outer zone of the maze on the first day of training. Interestingly, non-intubated control animals had the greatest path length in the outer zone, which was significantly higher than both intubated controls and alcohol-treated animals. This result is likely to be less related to spatial memory acquisition, as it is to anxiety, as thigmotaxis all but disappears once the animal starts to look for a hidden platform (Simon et al., 1994). This is a complex issue and will be discussed in the context of search strategies later in this section. When these data are normalised as a percentage of total path length the treatment group differences on day one are not significant, suggesting that they are not robust. There was a rapid decline between day one and day two in alcohol-treated animals percentage of path length in the outside zone and this can be seen in Figure 4.32. While this decrease in percentage of path length is not as dramatic in the combined control animals, this group sees further significant reductions between day two and day three and day two and day four, reaching a lower mean percentage.
of time spent in the outer zone by the fourth day compared to the alcohol-treated group. There were, however, no significant between group differences on any day, so repeating this experiment with a greater number of animals may elucidate differences between treatment groups. It must also be noted that within all groups (see 4.30) there was considerable variation in the path-length between animals for any one trial on any day. This is in part affected by some start positions (Vorhees and Williams, 2006) being much closer to the platform position, however it is also common to find a large range in path length in rats from 2 to 28 months in the Morris Water Maze reference memory, so increasing group size might be essential to detect group differences (Lindner, 1997).

The percentage of the path length that occurs in the quadrant, which contains the platform, while animals are learning the Morris water maze, is commonly used as a measure of performance (Gallagher et al., 1993; Kempermann & Gage, 2002). Surprisingly, there were no differences between treatment groups, both when three groups were analysed separately and when control groups were combined. Some subtle differences in performance within treatment groups in learning were identified between days of training, however. A lift in percentage path length in the target
quadrant was seen in both alcohol [p<0.0001] and control [p<0.001] groups between day one and day two, however controls [p<0.05] but not alcohols further improved between day two and day three. Percentage path length improvements were seen in both groups between day three and day four, however it was with a higher degree of significance in the control animals [p<0.0001] compared to the alcohol-treated animals [p<0.05]. These subtle differences in acquisition of reference memory are likely associated with the performance increase seen between day one and day two in the alcohol-treated animals with respect to thigmotaxis, above. With control groups combined, the latency to the platform showed this same trend. Alcohol animals showed marked improvement between day one and all other days [p<0.0001], however there was no further improvement from day two to day four with respect to latency to find the platform. On the other hand, combined controls latencies showed a more gradual improvement, with significantly reduced latencies between day two and day three [0.05] and day two and day four [0.0001]. The latency to the platform is included in this study for comparison to the literature; however, it is not as sensitive as path length because it can be influenced by rats’ swimming speed and by search strategy when the search includes the brief cessation of swimming to look around and presumably assess location against
the distant visual cues. In this case, swimming speed was not significantly different between treatment groups with all showing a gradual but significant decline in swimming speed as platform location memory was reinforced (Alaei et al., 2008).

Previous work investigating the effect of adult (Obernier et al., 2002) and adolescent (Schulteis et al., 2008) alcohol exposure on adult reference memory learning has produced similar results, with alcohol-treated animals performing similarly to controls with respect to Morris water maze learning. Interestingly, this has been demonstrated in the presence of significant alcohol neurodegeneration in the entorhinal and perirhinal cortices and in the dentate gyrus (Obernier et al., 2002), despite these being identified as key structures involved in normal water maze function (D’Hooge and De Deyn, 2001). Importantly, there was no significant neuronal degeneration found in these areas in the current study.

Following Morris water maze training was the probe trial, which tests memory of the platform location after a predetermined interval, in this case 24 hours to test long-term, reference memory. The probe trial, as mentioned previously, does not have a platform; therefore animals search for 60 seconds and their movements in relation to the
platform location are monitored. The path length and percentage of total time in each quadrant did not reveal any significant differences between treatment groups. Our hypothesis, that there would be a treatment effect, was not supported. Previous work using adolescent alcohol exposure and adult water maze behaviour, albeit only five days post the exposure interval, also revealed no significant differences in these measures between alcohol-treated and control animals on the probe trial (Schulteis et al., 2008). In order to further investigate any subtle differences between treatment groups in the probe trial, a number of further measures were investigated (Maei et al., 2009). Path length in the platform zone (a 10 cm radius circle around the centre of the previous location of the platform) could be considered more accurate than path length in a quadrant for measuring a search close to the platform. However, this measure again revealed no significant difference between the three treatment groups. The number of times the previous platform location was crossed, an oft-used performance measure (D’Hooge and De Deyn, 2001, Maei et al., 2009) also revealed no significant group effect. When control groups were combined there seemed to be fewer crossings in the alcohol group, however this difference was not statistically significant [p=0.0851]. Unsurprisingly in light of the above, there was also no effect of treatment on average proximity
(Gallagher et al., 1993) to the platform over the 60-second trial, this measure being the most reliable for identifying between group differences (Maei et al., 2009).

Research suggests that unlike mice, rats rapidly stop searching in a previous platform location when a new platform is introduced (Vorhees and Williams, 2006). Therefore, it is possible that during a long probe trial, rats may be aware that the platform is no longer where expected and actively search throughout the whole arena (Maei et al., 2009) or attempt to escape. For this reason, the first five seconds of platform crosses was analysed for sex and treatment effects with controls combined. This revealed a significant interaction between treatment and sex \([p=0.0126]\), with post hoc analysis revealing significantly less crossing in the male alcohol-treated animals compared to male controls \([p<0.01]\). There was no significant difference between treatment groups in the female animals, nor was there a significant difference between males and females of either treatment group. This data suggests that alcohol was the cause of a male-specific reduction in platform crossings from three crossings in the control animals to one in the alcohol-treated animals over five seconds of probe trial searching. This very subtle difference in spatial memory performance may be related to the fact
that rat brain development has a long trajectory (Spear, 2000) and that during adolescence the stage of hippocampal development in males versus females follows a different temporal profile (Coleman et al., 1987).

Literature indicates that there are sex differences in Morris water maze performance, with young males generally outperforming young females, however these are dependent on test design (Perrot-Sinal et al., 1996; D’Hooge and De Deyn, 2001). Evidence suggests that these differences are absent in Long-Evans rats of advanced age (Bucci et al., 1995). Furthermore, it has been proposed that female versus male differences in acquisition are due to the greater persistence of stress-related peripheral circling seen in early trials in female animals (Perrot-Sinal et al., 1996). A significant effect of both sex and treatment was found on the duration animals spent in the outer zone of the water maze during the probe trial. Post-hoc analysis revealed significant differences between the female alcohol and control animals, and between the male and female alcohol-treated animals, as seen in Figure 4.49. The data suggest that thigmotactic behaviour in the probe trial is more common in female animals if they have had adolescent alcohol exposure, a novel finding. As the mean duration of thigmotaxis in this group is nearly
30 seconds, or half of the trial, this indicates that these animals are not problem-solving. As there is no escape offered by the wall of the maze and this behaviour normally occurs early in training and is short-lived (Vorhees and Williams, 2006) the occurrence in the probe trial is interesting. Thigmotaxis is associated with increased stress (Perrot-Sinal et al., 1996) and as the Morris water maze is considered a relatively stressful behavioural task, the discovery that there is no platform may elicit a stress response leading to this stress behaviour in the some female rats as observed in the open field test (Archer, 1975) and the Morris water maze (Perrot-Sinal et al., 1996). Previous research has demonstrated an increase in long-term stress response in rats (Taylor et al., 1982) and primates (Schneider et al., 2002) when alcohol is given prenatally and adolescent alcohol exposure causes increased anxiety-like behaviour in both short term (Gomez and Luine, 2014) after three weeks of abstinence (Slawecki et al., 2004). The aforementioned studies did not investigate sex differences, however females were used exclusively in some studies (Taylor et al., 1982; Gomez and Luine, 2014), as females have a greater responsiveness to the hypothalamic pituitary adrenal stress activation pathway.
In the current study, ANOVAs revealed no differences between treatment groups in reversal testing for total path length across days or previous platform crossings. When path length for each trial was investigated with controls combined, there was a significant difference between trial one and all other trials in the control group only. However, as there was no overall effect of treatment, and there were no between treatment group differences on single trials this result is not robust. This was surprising, as reversal is differentially affected to reference memory tasks and an alcohol effect on medium-term reversal learning has been detected in another study (Obernier et al., 2002). The current study differed in its use of adolescent animals, length of training in the water maze and alcohol treatment paradigm. Adolescent alcohol exposure has been shown to decrease performance on a spatial working memory task without affecting reference memory performance (Schulteis et al., 2008). Depending on experimental design, reversal and delayed matching to place in the Morris water maze can be very similar, with inter-trial intervals being the main difference. Furthermore, reversal learning is sensitive to damage to the prefrontal cortex (de Bruin et al., 1994) a region that is also strongly associated with working memory (Carpenter et al., 2000). The delayed matching to place task for testing working memory in the Morris water maze is very sensitive to disruption of
hippocampal function (Steele and Morris 1999; Ferbinteanu et al. 2003; Nakazawa et al. 2003; de Hoz et al. 2005; O’Carroll et al. 2006; Bast et al. 2009; Pezze and Bast 2012). In the current study, the absence of any significant differences between performance of the alcohol-treated and control animals in the reversal task suggests that alcohol did not impair performance. This was surprising, given that Schulteis and colleagues (2008) demonstrated a significant reduction in delayed matching to place performance in adolescent alcohol-treated animals when an interval of two hours was used between trial one and two. However, an interval of one hour showed no significant differences between alcohol-treated and control animals, suggesting that, if it were present, this difference in working memory would not have been detected in the current study.

Search strategy analysis was performed for the current study, and results show a progression from peripheral circling, which as stated constitutes a stress response, to a systematic search strategy and then direct finding, when the location is known. There were no significant differences between treatment groups, however the graphs suggest that ‘random searching’ was utilised more by alcohol-treated animals on days 3 and 4 compared to other groups. These results are difficult to quantify, being qualitative measures.
Chapter Four: Experiment Three

As stated earlier, in the introduction of this thesis, the ethanol molecule has been shown to affect adolescent experimental, hippocampal long-term potentiation (LTP) in vitro (Swartzwelder et al., 1995; Pyapali et al., 1999), and an LTP-like process is thought to be highly involved in memory formation (see Martin & Morris, 2002 for review). There has been extensive investigation on the effects of alcohol on LTP but not as much interest in a related aspect of synaptic modulation, long-term depression (LTD). Alcohol has been shown to inhibit LTD in CA1 neurons of the hippocampus consistent with its effects on the NMDA receptor (Izumi et al., 2005) particularly the Fyn phosphorylation of the NR2B subunit (Wang et al., 2007). This finding has also been demonstrated in the cerebellum, at the synapses between parallel fibres and Purkinje cells which are thought to be involved in motor learning (Belmeguenai et al., 2008). It is now understood that some behaviours are more dependent on LTD than previously thought. Novel objects in a spatial field induce LTD in the CA1 region of the hippocampus (Kemp & Manahan-Vaughan, 2012), and there is evidence that blocking CA1 LTD prevents novelty acquisition and the benefits novelty exploration has on long-term memory (Dong et al., 2012). Furthermore, prevention of LTD but not LTP impairs spatial memory consolidation (Ge et al.,
2010), while disruption of LTD impairs performance and LTD facilitation boosts performance of reversal learning in the Morris water maze (Dong et al., 2013). Long-term potentiation is vulnerable to disruption by the ethanol molecule in the adolescent brain, however it is not known what effect alcohol drinking during adolescent brain development might have on LTD. The current study did not investigate LTD, however, given the recent findings above, this is likely to be the subject of important future research.

One of the interesting findings in this work has been the observation of different sex effects across a number of experiments. There is conflicting evidence as to whether males or females are more susceptible to alcohol induced brain damage and behavioural deficits in both human (Hommer et al., 2001; Mann et al., 2005; Yonker et al., 2005) and rat data (Savage et al., 2000; Gomez and Luine, 2014). Generally, in human clinical data, female alcoholics have an equal (Mann et al., 2005) or greater degree (Hommer et al., 2001) of tissue damage than males, despite usually having less history of alcohol dependence. However, when exposure occurs during brain development, the different time courses of development for brain regions of males and females (Cahill, 2006) can influence alcohol’s effect. These time courses will likely mean
that males and females have different periods of vulnerability for different brain regions, and this may explain some of the variation seen in our data. In contrast, circulating sex hormones can confer reduced susceptibility in certain situations as will be explained below. Light, moderate and heavy drinking in non-alcohol dependent human samples is associated with an increase in females’ performance in episodic and semantic memory, whereas increased levels of drinking decreases males performance in spatial visualisation tasks (Yonker et al., 2005). The explanation offered by the authors of the Yonker et al. (2005) study was that biological actions of alcohol caused an elevation of oestradiol levels, through aromatisation of androgens, that decreased males’ performance in the spatial visualisation task. A biological system for decreasing task performance in males in response to alcohol, therefore, exists. However, in the case of our results pertaining to novel object recognition performance, performance is usually higher in female animals; this makes it difficult to explain our findings in relation to the relatively sparse literature on adolescent alcohol exposure-dependent behavioural sex differences. A simple, alternative explanation that could be offered is that oestrogen’s neuroprotective properties, which are well documented (see Behl and Holsboer, 1999 for review) and include properties as an antioxidant preserved
function by protecting certain structures from some of the harm caused by alcohol. Previous research that investigated adolescent alcohol exposure, has demonstrated decreased discrimination ratio in the novel object recognition test in alcohol-exposed rats, as well as increased apoptotic markers in the neocortex and hippocampus (Pascual et al., 2007). Furthermore, it was found that treatment with indomethacin (a non-steroidal anti-inflammatory drug) at the time of alcohol exposure ameliorated the novel object recognition deficits and the increase in apoptotic markers to levels consistent with control animals. It may be possible that in the current study, female animals’ frontal brain regions, that were responsible for working memory and novelty recognition, were protected from alcohol-induced inflammation by oestrogen during adolescent alcohol exposure. In contrast, male animals did not have this protection, and therefore experienced long-term damage caused by apoptotic cell death and subsequent inflammation that affected their performance on this task.
5 Conclusion

This thesis presents an animal model, modified from previously used models, to more accurately represent the binge mode of drinking seen in adolescence. This model was then used to study the effects that such drinking has on the brain and on behaviour. Several animal models have been described in the literature and are used to model adolescent binge drinking, as outlined in previous chapters. It is of extreme importance that the details of the human adolescent drinking paradigm must be integral to a model so that the model is as reliable as possible and thus can be considered to reflect the human condition. However, differences between these models, with respect to the results obtained from them and differences in methodology, are stark and more consideration must be given to the details of adolescent drinking patterns to accurately model this condition.

Intermittent binge alcohol consumption resulted in acute widespread brain damage using the Crews et al. (2000) model, where
the pattern of alcohol exposure resulted in animals remaining intoxicated for several days, which is unlikely to be relevant to the majority of alcohol-consuming adolescents. Furthermore, this model was developed to produce alcohol dependence, gene expression and brain damage in adults and its use as a model of adolescent binge drinking may be inappropriate. Another model, used by Pascual et al. (2007), has been used to demonstrate evidence of acute apoptotic cell death, inflammation of the brain and behavioural deficits following adolescent alcohol exposure. However, the alcohol dose was delivered via an i.p. injection and resulted in rapid intoxication and mean time to loss of consciousness of 90 seconds, something that does not happen in the human adolescent. Thus it fails to accurately model what is happening in the human adolescent. The model we used in the current study was developed to more accurately model the pattern, rate and volume of alcohol consumption, as well as produce a level of intoxication that is consistent with the data from binge-drinking adolescents. The age range used in the current study was chosen to model the equivalent age range in humans who were deemed most at risk in the current literature. In the current study, animals were sacrificed 24 hours after each dose and brains were surveyed for evidence of widespread apoptosis and gliosis. In contrast to the published literature there was no evidence of
significant apoptotic cell death or gliosis in the ethanol-treated group at any time. A qualitative survey suggested that there were more/thicker branched astrocytes in the anterior cingulate cortex in alcohol-treated animals. This was further investigated using unbiased stereology. This study showed that in fact there were significantly fewer astrocytes in this region in alcohol-treated animals. Unfortunately, this finding may have been affected by a different sex bias within each group studied, as the cingulate cortex may be a brain region that is sexually dimorphic with respect to astrocyte density. This finding was unexpected, as neuro-inflammation, is usually accompanied by pronounced astrocyte activation. Micrographs from the current study do not suggest astrocyte activation in the alcohol-treated group.

The current study was unique in that the behavioural testing was performed after an extended period of abstinence in order to specifically look at the long-term effects of adolescent alcohol exposure. Animals exposed to alcohol between PN28 and PN48 underwent behavioural testing at around one year of age, thus any differences observed can be attributed to changes in brain structure as a result of alcohol exposure or as a result of alcohol-induced brain changes inducing structural changes during development up to the
age of death. This design removes the possibility of a developmental delay being the causation of change when analysis occurs at PN60, shortly after alcohol termination, but also allows for any compensatory plasticity that may occur as the brain, during adolescence and early adulthood attempts to compensate for alcohol-induced damage. The main finding in the behavioural component of the current study was that, despite prolonged intermittent binge alcohol exposure during adolescent development, there were almost no discernable differences between alcohol-treated and control animals. The range of behavioural tasks used assessed the function of brain regions that had previously shown alcohol-induced deficits following adolescent alcohol exposure. The fact that there were no significant effects of treatment on most measures suggests that in this study, the brain revealed a remarkable ability to cope with the repeated insult that is known to occur with intoxicating levels of alcohol. Studies of AUD teenagers have shown that while function can be considered normal, structure has been changed in response to alcohol use (Caldwell, 2005).

Interesting and important findings related to behavioural testing in this study include: the performance deficit in male, alcohol-treated animals in the novel object recognition task; the early platform zone
finding deficit in male alcohol-treated animals performing the probe trial of the MWM; and the thigmotactic response of female alcohol-treated animals to the probe trial. These results demonstrate the type of subtle changes in behaviour, persistent over a long-time that one might expect from repeated binge drinking during adolescent brain development. These findings in the adult, although minimal, could have resulted from behavioural and anatomical plasticity occurring from adolescence to adulthood to ameliorate a greater deficit visible soon after the exposure period. If these results correspond to the impact of adolescent binge drinking on the human brain, the damage that may be being done, which may not manifest itself until later in life, although minimal will impact on brain function and is thus alarming.

5.1 Future Research Directions

This study, despite some negative results and no-change findings, has made an important contribution to our understanding of the impact of repeated binge drinking on adolescent brain development. As this behaviour is increasing, and has become a worldwide problem, it is important that animal models, that as accurately as possible reflect human drinking patterns, are used so that we can
determine what may be happening to the brains of an increasing number of adolescents. This study has pointed out that much of the published research is inadequate and may in fact be incorrect in illuminating adolescent alcohol consumption brain damage. The lack of ‘significant differences’ in the current study may actually be a more accurate reflection of what occurs. This data should not be used to support adolescent binge drinking but to stress that the animal model used must reflect as accurately as possible the conditions being modelled.

The gavage method, although commonly used was a somewhat stressful experience for the adolescent animal, probably as alcohol must be delivered in a larger volume than other drugs. However, the vapour inhalation method could be used in future studies, despite the ‘non-physiological’ mode of delivery, allowing a model using a realistic rate of increase in blood ethanol concentration and periodic exposure to advance knowledge in this area. Using this method of delivery, the key area to be studied is very early adolescence in the rat, PN28, as prospective human data has shown that the age of onset of alcohol consumption is the key factor in negative life outcomes in adult life (Odgers et al., 2008)
The alcohol-related reduction in astrocyte count in the rostral cingulate gyrus needs to be further investigated. Reduction of astrocyte numbers can have an effect on the health of other cells in the brain region and would impact on brain ageing. A stereological investigation of total neuron and glial cell number would detect any long-term changes. With the detection of only subtle changes at one year it is important to ascertain whether there are behavioural deficits in early adult life, thus helping to elucidate whether the changes at one year represent a reduction of early behavioural deficits or indicate early aging related changes. Due to considerable variation in the data, future studies need to use a larger number of animals per group. If even only one in 100 adolescents that binge drink during adolescence suffer long-term brain damage, this would be a considerable burden to both the individual and society.

This study has clearly shown that the results obtained in any study are dependant on the model used and thus having achieved a more realistic adolescent-alcohol model that challenges whether/or how, earlier data can be extrapolated to the human condition, this model should now be used in a range of studies. These could include identifying the relationship between LTP, LTD and alcohol’s effects on developing brain networks and behaviour. The function of neurons and hence neuronal circuits, can be affected by much
smaller structural changes than cell death or gliosis. For this reason it is essential to parallel structural and behavioural analysis with electrophysiological investigations. Any electrophysiological change will impact on function and will inform on what cellular or molecular changes should be investigated.

Despite the paucity of significant changes in this study, it does provide strong evidence that models must accurately reflect the behaviour they are attempting to model and that much research is still needed to understand the potential negative effect of both intermittent binge drinking during adolescence and also the early onset of this binge drinking behaviour as noted by Odgers et al. (2008).
References


Chapter Six: References


Flores, C., Salmaso, N., Cain, S., Rodaros, D., & Stewart, J. (1999). Ovariectomy of adult rats leads to increased expression of astrocytic basic fibroblast growth factor in the ventral tegmental area and in dopaminergic projection regions of the entorhinal and prefrontal cortex. *The Journal of Neuroscience, 19*(19), 8665-8673.


Chapter Six: References


Appendices
Appendix A: Paw and Ear Marking

To identify animals, paw or ear marking was performed on PN6. With a single ear punch, rats could be numbered 1-6. Paw marks could be used to number up to 13. Multiple earmarks or pawmarks were combined to make higher numbers.

Appendix B: Details of Animal Deaths

Records of animals that died during this study are as follows in the chart below. In a single litter, three animals did not recover following intubation from alcohol-induced intoxication leading to coma. It appears these animals received an extra alcohol intubation. Also in this litter on the same day, three rats were perfused between
7.5 and 9 hours post intubation as the behavioural indicators suggested they would not recover. Some rats from both treatment groups were euthanized while in the HTRU awaiting behavioural testing. Cause of death was not determined for these animals, however, it was unlikely to be related to experimental treatment.

<table>
<thead>
<tr>
<th>Date</th>
<th>Litter</th>
<th>ID</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/5/2010</td>
<td>3</td>
<td>F7</td>
<td>misintubated, dose 1</td>
</tr>
<tr>
<td>20/5/2010</td>
<td>7</td>
<td>F15</td>
<td>misintubated, dose 2</td>
</tr>
<tr>
<td>28/5/2010</td>
<td>6</td>
<td>M1</td>
<td>unknown, 4 hours post intubation</td>
</tr>
<tr>
<td>28/5/2010</td>
<td>6</td>
<td>M5</td>
<td>unknown</td>
</tr>
<tr>
<td>28/5/2010</td>
<td>6</td>
<td>M4</td>
<td>perfused, unconscious 9 hours post</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>intubation</td>
</tr>
<tr>
<td>28/5/2010</td>
<td>6</td>
<td>M6</td>
<td>unknown, 8 hours post intubation</td>
</tr>
<tr>
<td>1/6/2010</td>
<td>7</td>
<td>M3</td>
<td>perfused, 7.5 hours post intubation</td>
</tr>
<tr>
<td>1/6/2010</td>
<td>7</td>
<td>M9</td>
<td>perfused, 8 hours post intubation</td>
</tr>
<tr>
<td>5/6/2010</td>
<td>6</td>
<td>F4</td>
<td>misintubated, dose 1</td>
</tr>
<tr>
<td>29/7/2010</td>
<td>8</td>
<td>M3</td>
<td>misintubated, dose 1</td>
</tr>
<tr>
<td>6/8/2010</td>
<td>8</td>
<td>F2</td>
<td>misintubated, dose 3</td>
</tr>
<tr>
<td>10/8/2010</td>
<td>8</td>
<td>F3</td>
<td>misintubated, dose 3</td>
</tr>
<tr>
<td>11/8/2010</td>
<td>9</td>
<td>Fblue3</td>
<td>misintubated, dose 1</td>
</tr>
<tr>
<td>14/8/2010</td>
<td>8</td>
<td>M6</td>
<td>misintubated, dose 1</td>
</tr>
</tbody>
</table>
## Appendix C: Daily Dose Calculation Sheet

<table>
<thead>
<tr>
<th>Animal Code</th>
<th>Male/Female</th>
<th>Weight</th>
<th>Date</th>
<th>Total Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Calculation:**

Total Dose = \( \text{Total Gavage} \times \text{Total Dose per gavage} \)
Appendix D: Internal Standards for Alcohol Analysis

The internal standard solution is a solution of 50µl of n-propanol in 100ml of 1% sodium fluoride containing sodium azide (preservative). Ethanol standard solutions are made up from a stock ethanol solution of 20% by weight (20% ethanol solution is 20,000mg/100ml).

The standards are:
1. 500ul stock in 100ml of saline is 78mg/dl
2. 1000ul stock in 100ml saline is 158mg/dl
3. 1.5ml in 100ml saline is 237mg/dL
4. 2ml in 100ml saline is 316mg/dl
5. 2.5ml in 100ml saline is 395mg/dl

To make up the standard stock ethanol solution:
Use top loading balance,
- Measure out 50ml of water,
- Put in glass flask add 100% ethanol from pipette until 20g of ethanol has been added by weight
- Make up to a final total volume of 100ml with double distilled water
Samples: Use a 1:9 ratio, 1 volume of blood or alcohol standard and 9 volumes of internal standard i.e. 20μl of blood and 180μl of internal standard. Replace the 20μl of blood with 20μl of an ethanol standard for the ethanol standard series.

Appendix E: Cresyl Violet Staining Protocol

Mounting Sections
Section on cryostat (thickness 40μm)
Wet mount in dH2O
Use doube subbed slides
Put 4-5 sections on a slide

Solutions
0.1% Cresyl Violet Solution
70% and 95% acetyl EtOH
Add 12 drops of Glacial Acetic Acid for every 250mL of EtOH to make acetyl EtOH

Method (all steps done in glass staining boats)
Chapter Seven: Appendices

1. Put sections in water for 30 sec
2. Stain in Cresyl violet solution for 25 min (covered)
   a. filter the Cresyl violet before each use
3. Put in water for 30 sec
4. Put in 70% acetyl EtOH for 3 min
5. Put in 95% acetyl EtOH for 3 min
6. Put in 100% EtOH for 3 min
7. Put in 100% EtOH for 3 min, again (use fresh EtOH)
8. Put in Xylene for 10 min
   a. take slides out of Xylene one at a time to coverslip so that they don’t dry out.

To make 300 mL of 0.1% Cresyl Violet

1. Add 5mL of 1% Cresyl Violet Stock solution to 45mL dH₂O ➔ makes 50mL 0.1% Cresyl Violet

Make 250mL of buffer solution (3 parts 0.2M acetic acid + 2 parts 0.2M sodium acetate)

2. Add 18mL of acetic acid to 132mL of dH₂O ➔ makes 150mL of 0.2M acetic acid.
   60g/L acetic acid = 1M acetic acid
0.12L acetic acid + 0.88L dH₂O = 1L 0.2M acetic acid

3. Add 1.641g sodium acetate anhydrous to 100mL dH₂O ☐ makes 100mL 0.2M sodium acetate
   82.03g sodium acetate anhydrous/1L = 1M sodium acetate

4. Add the 0.1% Cresyl Violet solution to the buffer solution.

   Final Cresyl Violet = 0.025%

Appendix F: Cutting Regimen for Second Phase of Histology

1. Set Cryostat to cut at 40 microns.
2. Find start section, the sections will look like this:

3. For medial and ventral orbital cortices take sections: 3, 4, 5, 6, 10, 11, 12, 13, 17, 18, 19, 20, 24, 25, 26, 27, 31, 32, 33, 34, 38, 39, 40, 41, 45, 46, 47, 48

4. For anterior cingulate gyrus take sections: 21, 22, 23, 24, 39, 40, 41, 42, 57, 58, 59, 60, 75, 76, 77, 78, 93, 94, 95, 96, 111, 112, 113, 114, 129, 130, 131, 132
5. For dentate gyrus and CA3 take sections: 147,148,149,150, 162,163,164,165, 177,178,179,180, 192,193,194,195, 107,108,109,110, 122,123,124,125, 137,138,139,140
   a. or same sections as dentate gyrus and CA3
7. 28 stops total and 112 sections. Need 3 cell culture plates per brain. There are 17 brains so need 51 cell culture plates. (OR use sections for both amygdala and hippocampus and they can share the same ‘stops.’ Would mean 84 sections rather than 112.)
8. Cut all brains and put sections into cryoprotectant.
9. Stain 4 stops at a time from all brains, 68 sections, two staining trays hold 72 sections so there is space for controls.
10. Two immuno runs needed to complete each AOI. 8 large immuno runs to complete the staining for each antibody. 24 large immuno runs needed and one CV run. (OR if sharing sections 17 brains 72/17=4.2, 8 GFAP runs, 1 CV run and 8 IsoB4 runs.)