Drinking During the Third Trimester of Pregnancy – a Cause for Concern

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Abstract

Introduction: Foetal Alcohol Spectrum Disorder (FASD) is an array of disorders attributed to CNS damage, behavioural and social manifestations after varying degrees of prenatal exposure to alcohol. Due to the majority of effects being phenotypically subtle, many cases are not diagnosed, and subsequently do not receive support and treatment. Drinking during pregnancy is an on-going issue in New Zealand, in particular binge-like episodes resulting in high blood alcohol concentrations. These episodes have been shown to continue throughout pregnancy in some women, and a lack of evidence correlating specific structural and functional outcomes after a single alcohol binge during the third trimester leaves room for question about what timing and quantity of alcohol induces lasting abnormalities in early life and adulthood.

Methods: Two cohorts of animals were used – (1) for stereological studies and (2) for behavioural studies. On Post Natal day 6 (PN6), the human third-trimester equivalent, male and female Long Evans rats were randomly assigned into one of three treatment groups and administered an intragastric intubation; Ethanol 6.0g/kg (E6), 5.25g/kg (E5), 4.5g/kg (E4); and two control groups - Intubation control (IC) and Suckle Control (SC). Cohort 2 underwent social play behaviour analysis via video recording from P32-34, and again on PN80-82. Animals were later analysed on the Elevated T-maze (ETM) for anxiety-related responses to an aversive environment. Cohort 1 were deeply anaesthetised and perfused on PN365. Brains were removed, cryoprotected, frozen and sectioned in the coronal plane at 60µm. A random systematic set of sections were stained with thionin followed by unbiased stereological methods using the optical disector to determine the number of neurons in the Anterior Cingulate Cortex (Acc) and hippocampal CA1 area.

Results: Cohort 1 revealed a dose-dependent loss of hippocampal CA1 cells across all alcohol-exposed groups (P<0.01); along with E6 and E5 animals showing a 39.6% and 30.8% (P<0.05) mean decrease in Acc cells relative to IC animals. The E6 group also had significantly less hippocampal CA1 and Acc neurons than the E4 group (P<0.05). Play behaviour analysis in cohort 2 revealed increased rates of attack by the alcohol-exposed and IC groups compared with controls (P<0.05). SC animals did not display the normal reduction in attack frequency between adolescence and adulthood, while E6 animals preferred the evasive form of defence significantly more often than all other
groups during adulthood (P<0.05). ETM testing did not reveal any definitive anxiogenic / anxiolytic effects of prenatal alcohol exposure (P = 0.058), however E6 animals showed a significant lack of response inhibition from trial 1 to trial 2 on the inhibitory avoidance task relative to IC and SC animals (P<0.05). The one-way escape task revealed E6 animals took significantly less time than E4 animals to escape into the closed arm, however as no difference was found with relation to control groups this result was equivocal.

**Conclusion:** It is clear that a single ethanol binge on PN6 induces significant neuronal death in both the hippocampal CA1 (E6 = 49%, E5 = 38%, E4 = 26%) and Acc (E6 = 28%, E5 = 24%, E4 = 5%) areas, in a dose dependent fashion. Both of these areas are involved with social functioning, and corresponding social play abnormalities were observed, however not in the fashion hypothesized. During adolescence, both ethanol-exposed and control groups were behaving abnormally and showing different rates of defensive patterns than previously reported in the literature. This may be due to a “rub off” effect of the alcohol animals being housed with the control animals. These social play behaviour anomalies seen in adolescence were not evident in adulthood, indicating some level of functional recovery to appropriate levels of attack and defence during a ten-minute play period. However E6 animals showed a significantly higher probability of evading an attack than SC animals, suggesting a preference for bout termination possibly due to intimidation by the more cognitively intact SC animals. Anxiety testing revealed anomalies in response inhibition in E6 animals, however further refined studies are needed to definitively elucidate these putatively subtle changes.

These data contribute to the growing pool of evidence that any amount of alcohol during any stage of pregnancy may have serious repercussions, and only through continuing research will we begin to build an evidence base for public health policy and guidelines.
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<th>Description</th>
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<tbody>
<tr>
<td>Acc</td>
<td>Anterior cingulate cortex</td>
</tr>
<tr>
<td>Acd</td>
<td>Anterior cingulate cortex dorsal</td>
</tr>
<tr>
<td>Acv</td>
<td>Anterior cingulate cortex ventral</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention deficit hyperactivity disorder</td>
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<tr>
<td>AEC</td>
<td>Animal ethics committee</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ARND</td>
<td>Alcohol related neurodevelopmental disorder</td>
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<tr>
<td>asf</td>
<td>Areal sampling fraction</td>
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<tr>
<td>BAC</td>
<td>Blood alcohol concentration</td>
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<tr>
<td>BEC</td>
<td>Blood ethanol concentration</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis area 1 of hippocampus</td>
</tr>
<tr>
<td>CA2</td>
<td>Cornu Ammonis area 2 of hippocampus</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis area 3 of hippocampus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
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<tr>
<td>DPX</td>
<td>Distrene, plasticiser, xylene</td>
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<tr>
<td>E4</td>
<td>4.5/kg Ethanol-treatment group</td>
</tr>
<tr>
<td>E5</td>
<td>5.25/kg Ethanol-treatment group</td>
</tr>
<tr>
<td>E6</td>
<td>6.0/kg Ethanol-treatment group</td>
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<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
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<td>ETM</td>
<td>Elevated T-Maze</td>
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<td>EtoH</td>
<td>Ethanol</td>
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<tr>
<td>FAE</td>
<td>Foetal alcohol effects</td>
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<td>FAS</td>
<td>Foetal alcohol syndrome</td>
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<td>FASD</td>
<td>Foetal alcohol spectrum disorder</td>
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<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Generalized anxiety disorder</td>
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<tr>
<td>GD</td>
<td>Gestational day</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IC</td>
<td>Intubation control</td>
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<tr>
<td>LMM</td>
<td>Linear mixed model</td>
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<tr>
<td>mPFC</td>
<td>Medial prefrontal cortex</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MWM</td>
<td>Morris water maze</td>
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<tr>
<td>OFC</td>
<td>Orbito frontal cortex</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PD</td>
<td>Postnatal day</td>
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<td>PD</td>
<td>Panic Disorder</td>
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<tr>
<td>PE</td>
<td>Polyethylene</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
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<tr>
<td>Rsc</td>
<td>Retrosplenal cortex</td>
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<tr>
<td>SC</td>
<td>Suckle control</td>
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<td>ssf</td>
<td>Section sampling fraction</td>
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Chapter 1. Introduction

Foetal alcohol syndrome (FAS) is a neuropathological condition which can devastate the central nervous system (CNS), and affect many aspects of development that arise in the foetus as a result of maternal drinking during pregnancy (Jones and Smith, 1973).

FAS is characterised by varying levels of craniofacial malformations (Figure 1.1) which can include a smooth philtrum, widely spaced eyes, short palpebral fissures, a thin vermilion border of the upper lip, severe pre and post-natal growth retardation, microcephaly and various CNS disorders (Jones and Smith, 1973, Sulik et al., 1981, Sulik, 1984, Schenker et al., 1990, Jones et al., 2006). Classical FAS (Figure 1.2) can only occur early during the very limited period of development in the first trimester of pregnancy, during the morphological development of the face and early CNS (Schenker et al., 1990). Therefore any exposure to alcohol after this period is unlikely to affect the structure of the craniofacial makeup.

Figure 1.1 Craniofacial anomalies attributed to FAS

Children from different ethnic backgrounds, as seen from left to right - Asian, African American and Caucasian all show the characteristic facial dysmorphology that can be directly attributed to FAS. Adapted from Jones and Smith (1973).
While FAS has been recognised in most developed countries for over 30 years, Foetal Alcohol Spectrum Disorder (FASD) is an umbrella term, which has only become clinically recognized in recent years (Mukherjee et al., 2006). FASD encompasses the well known FAS, however through various lines of research many other alcohol-related syndromes are being described and classified into the FASDs. These include alcohol-related neurodevelopmental disorder (ARND), alcohol related birth defects (ARBD), foetal alcohol effect (FAE) and partial foetal alcohol syndrome. FASD in itself is not a diagnosis – however more so describes the possible range of complications that may arise after maternal consumption of alcohol. Many children with documented exposure to some quantity of prenatal alcohol do not meet the diagnostic criteria of FAS, and as a result cannot receive a diagnosis and subsequent clinical intervention. To date human studies have had to rely on retrospective and post-mortem data for obvious ethical reasons, therefore this PhD examined an animal model of FASD – and investigated neuronal structural and behavioural changes in rats exposed to ethanol on a single day in the third trimester equivalent.

**Figure 1.2 Faces in FAS**
This figure depicts both the overt and subtler features that may present in a child with classical FAS. All of these features may be present, or select few depending on the level of exposure and timing of gestation during which the insult occurred. Figure adapted from Streissguth and colleagues (1994).
Although the majority of deficits may be phenotypically subtle, an array of neurodevelopmental, cognitive and behavioural anomalies may exist which show significant deficits over alcohol exposed children that do however not have a clinical diagnosis of FAS (Streissguth et al., 1994, Mattson et al., 2001, Riley et al., 2003). After the first trimester, following the development of the neural tube and subsequent formation of the essential structures of the CNS, CNS development continues throughout the remainder of gestation. Consumption of alcohol during this period can result in altered CNS development and can manifest a lesser form of alcohol induced damage, characterised by a variety of subtle, yet debilitating CNS deficits. FASD is associated with learning and memory impairments, attention deficit hyperactivity disorder (ADHD) (O'Malley and Nanson, 2002b), decreased attention span, motor abnormalities and lack of social inhibition in juveniles, and various states of psychosis or depressive episodes during adolescence and adulthood (Wozniak et al., 2004, Rasmussen et al., 2006).

Correlation studies have shown that early in uteri exposure to ethanol during the first trimester predominantly results in craniofacial abnormalities and severe mental deficits associated with FAS. However exposure in the third trimester (in humans) during the brain ‘growth spurt’ period of synapse formation, generation of small neurons and the beginning of glial cell proliferation, causes major deficits related to learning and memory – a common aspect of FASD (Sokol et al., 2003, O'Leary-Moore et al., 2006).

1.1 Human studies

Tests of explicit and implicit memory on subjects with prenatal exposure to alcohol show disparate disruptions between the two, demonstrating non-globalised effects, as these two memory systems are structurally and functionally distinct entities. Implicit memory – the unconscious innate long term memory of skills or procedures, was unaffected in the alcohol exposed subjects measured through a priming test, while impairment was shown in tests measuring declarative or explicit memory in a free recall task (Mattson and Riley, 1999). Further studies have revealed that alcohol affected children perform similar to control children on a test of verbal learning (implicit), while showing significant deficits on a test of non-verbal (explicit) learning and memory. The use of an implicit strategy positively affected the ability of alcohol-affected children to
learn and retain new verbal information. This data indicates that alcohol-induced changes in brain function can be very specific and it may be difficult to link the deficit to prenatal alcohol exposure unless a detailed history of maternal drinking behaviour is available (Roebuck-Spencer and Mattson, 2004). Structural deficits on both a gross anatomical and cellular level are also present in children and adults exposed to some level of alcohol prenatally.

Magnetic resonance imaging (MRI) studies in children with a variety of FASDs have shown a region – specific pattern of dysmorphology namely in the parietal, frontal and posterior temporal lobes (Sowell et al., 2007). This dysmorphology is presented as an increase in cortical thickness. This may seem counter intuitive, however studies have shown that normally developing children show a reduction in thickness of these cortices during development into adolescence which may not be occurring in the alcohol-exposed children (Sowell et al., 2004). MRI studies have also found deep brain structural abnormalities in children, teenagers and adults that have been diagnosed with FAS. Anomalies of the brain midline, including agenesis of the corpus callosum, enlargement of the ventricles and separation of the leaflets of the septum pellucidum (Cavum vergae) indicate the extreme vulnerability of the midline structures to the teratogenic effects of alcohol. These midline anomalies were also correlated with concurrent dysmorphology of the craniofacial structures mentioned earlier (Swayze et al., 1997). These behavioural and MRI studies provide a wealth of information relating to the damage caused by prenatal drinking, however there is a paucity of literature relating to the actual level and duration of alcohol consumption by mothers during at-risk pregnancies.

A New Zealand study investigating nutrition during pregnancy unveiled alarming statistics regarding maternal drinking patterns, type of alcohol and volumes consumed (Watson and McDonald, 1999b). Drinking statistics were collected, as part of a nutritional study, from a group of pregnant New Zealand women during the second trimester of pregnancy, who were selected on the basis of their geographic location. After knowledge of conception, 29% of the total sample (144 subjects) still drank on a regular basis – and 10% (15 subjects) continued to drink to intoxicating levels while pregnant. The data indicated that none of the 144 women who continued to drink during pregnancy, from the original 409, drank each day – suggesting that typical binge drinking during pregnancy is not likely to occur on consecutive days (Watson and
McDonald, 1999a). A subgroup of at least six women was shown to drink at binge levels of greater than 100g of alcohol per drinking session that would produce a high peak blood alcohol concentration (Figure 1.3). In New Zealand, the definition of “binge levels” is more than six standard drinks for men, and more than four standard drinks for women on one drinking occasion (Ministry of Health, 2009). As one standard drink contains 10 grams of alcohol, the exposure of these women to levels equivalent to 10 standard drinks poses a significant threat to the foetus, as exposure to ethanol at 200mg/dl for more than 4 hours has been shown to result in foetal damage in controlled animal studies (Ikonomidou et al., 2000).

**Figure 1.3 Alcohol consumption during pregnancy**

A depiction of the total alcohol intake of a number of high-risk women during one drinking session during pregnancy in New Zealand. Adapted from (Watson and McDonald, 1999b).

Controlled animal studies, such as that by Ikonomidou et al (2000) have, however led to masses of reliable, reproducible data on the cause of neurodegeneration due to foetal exposure to alcohol. The dose-response correlation and repercussions on cognition, behaviour and motor outcomes are beginning to be pieced together in this complex and often overlooked area of preventable pathology. Animal studies are essential in this
field, which will be shown below, as the lack of reliable data on the drinking patterns of individual women who have children with a diagnosis of FAS or FASD makes interpretation of the data difficult and sometimes misleading. Such studies may also be able to elucidate a pattern of behavioural disabilities that is suggestive of prenatal alcohol exposure and is thus of diagnostic importance.

### 1.2 Advantages of animal studies

Studies conducted with human subjects are extremely important for diagnostic purposes, behavioural intervention strategies and preventative measures. However the strength, accuracy and therefore validity of such studies can be placed in doubt as so many confounding factors exist that cannot be controlled due to ethical restrictions (Mukherjee et al., 2006). The timing and dose information of alcohol consumption by the mother is usually acquired through retrospective analysis, which cannot be accurate to the point that comparisons could be made between similar studies with temporal consumption and or dose data leading to behavioural impairments (Weinberg, 1996).

Other factors such as smoking and/or drug abuse would further confound results and as above the precise dose could not be accurately obtained. Social/habitual settings and varying methods of upbringing by caregivers also confound these studies. Tests of learning, memory, behaviour and cognitive abilities have also yet to be standardized throughout human studies and as a result comparisons and meta-analysis of data is one aspect that is difficult to undertake. Animal models however allow the researcher to administer the ethanol at precise doses at equivalent times in development between species, including at times comparable to human foetal development. The amount of data available from animal studies due to large litters, short gestation periods and decreased life spans can result in increased significance of findings, and shorter periods through which the study can be successfully carried out.

The concept of controls is also an important factor in any experiment, and it is sometimes difficult to gain adequately matched control groups in human studies. In animal studies treatment can be identical – that is matched for age, strain, parity, initial body weight and sex – the only difference would be the administration of alcohol as a variable, therefore any effects seen can confidently be attributed to the administration of
alcohol (Zajac and Abel, 1992). The requirement for animal models in this field is therefore essential for determining set periods of vulnerability, a dose-dependent relationship, and behavioural and neuroanatomical changes that occur as a feature of FASD.

Numerous animal studies have shown that the third trimester equivalent of CNS development is a period of teratogenicity for alcohol and that a short binge exposure to ethanol is enough to produce long term functional deficits and structural brain changes (Kelly et al., 1987, Goodlett et al., 1989, Bonthius and West, 1991, Goodlett et al., 1991, Goodlett et al., 1993, Napper and West, 1995, Pauli et al., 1995a, b, Goodlett and Johnson, 1997, Goodlett et al., 1998, Thomas et al., 1998b, Tomlinson et al., 1998, Tran and Kelly, 2003, O'Leary-Moore et al., 2006, Klintsova et al., 2007, Lewis et al., 2007, Shoemack, 2007). It is thus important to assess the effect of a single binge episode occurring within a single day in an animal model to elucidate the potential risk of binging on non-consecutive, frequently widely spaced days in the pregnant human.

1.3 Animal studies of FAS / FASD

Initially it was crucial to show that alcohol is teratogenic, and this was the perfect beginning for the incorporation of an animal model. A seminal study by Sulik, Johnson & Webb (1981), showed that small doses of ethanol administered four hours apart on gestational day 7 (human first trimester equivalent) produced distinct craniofacial malformations synonymous with human FAS. Following this study, animal models of both pre and postnatal exposure – separately and combined – to ethanol have been used by researchers worldwide to investigate the temporal windows of vulnerability of the brain to the ethanol and the dose-dependent relationship with respect to structural and functional abnormalities. Various neuroanatomical studies have begun to clarify the specific pattern of cellular damage within cortical and sub-cortical regions of the developing brain and the long-term changes following exposure to ethanol during development (Crews et al., 2004).
1.3.1 Periods of vulnerability to ethanol

Ethanol has been shown to induce cell-death via apoptotic mechanisms, and therefore acute analysis can identify dying cells either via activation of caspase-3 or by detection of the changes in cell morphology, neurotrophic factors and proteins that are characteristic of apoptosis (Dikranian et al., Zhang et al., 1998, Dikranian et al., 2001, Light et al., 2002, Olney et al., 2002a, Olney et al., 2002c, Heaton et al., 2003, Tenkova et al., 2003). The early neonatal period in rats is a period of vulnerability for ethanol-induced neurotoxicity across a number of brain regions. Early work investigating the effects of neonatal ethanol exposure on the cerebellum revealed this region to be highly susceptible to apoptotic cell loss during the third trimester equivalent and subsequent functional decline (Napper and West, 1995, Klintsova et al., 1997, Goodlett et al., 1998, Light et al., 2002, Siler-Marsiglio et al., 2005). In the hippocampus, ethanol exposure predominantly affects the CA1 pyramidal neurons currently undergoing differentiation (Bonthius and West, 1990, Livy et al., 2003, Tran and Kelly, 2003, González-Burgos et al., 2006). These neurons are post-mitotic, and therefore are unable to regenerate after undergoing apoptosis (Klintsova et al., 2007). Livy et al (2003) showed that CA1 pyramidal cell density was selectively depleted after a binge exposure to ethanol only during post-natal days 4-9 (PN 4-9) (third trimester equivalent). CA3 pyramidal neuronal number was also affected, however not to the same extent, and exposure during either the first or second trimesters or both revealed no significant deficit in either CA1 or CA3 pyramidal cells (Livy et al., 2003). This lends evidence to a temporal window of vulnerability to ethanol in the hippocampus during development, and as cellular aspects of the hippocampus are involved in the deficit this implies that the main hippocampal function of spatial learning and memory are likely to be affected also.

Various groups have also investigated the specific time periods within the brain growth spurt during which ethanol exposure can elicit behavioural impairments, and the dosing regimens that produce deficits. A landmark study was conducted in 1997 by Goodlett & Johnson, which investigated the timing and dose effects of a daily binge ethanol exposure on place learning in the MWM. They used a paradigm of varying doses of ethanol, which was important in elucidating the dose-dependent effects of ethanol on these behavioural tasks. The first experiment involved a dose of 5.25 g/kg/day
delivered in two intubations per day on PN 4-6, PN 7-9 or PN 4-9 – producing average peak blood alcohol concentrations (BACs) of 265 mg/dl. Deficits in place learning on PN 26-31 were only found in animals exposed to ethanol on PN 7-9 or PN 4-9, not on PN 4-6. Experiment 2 involved two groups of rats, one given a dose of 4.5 g/kg/day, the other a dose of 5.25 g/kg/day on PN 7-9. Only the higher dose resulted in place learning deficits; however both dose groups showed deficits in localization in the probe trial, where the submerged platform was removed. This study indicates a temporal window of vulnerability to spatial learning deficits localized to the second neonatal week, where only 2 consecutive days of exposure are required to produce spatial learning deficits. The severity of the deficit suggests that a single day binge on one of these vulnerable days may lead to similar deficits in spatial learning and memory (Goodlett and Johnson, 1997). This study tested animals on PN 26-31 – as they are entering adolescence, and thus on-going plastic changes are still occurring in the brain as they are entering adolescence. This study may not represent the entire story as performance may improve as the animals mature, beyond PN 31, as PN 31 falls in a period of rapid brain development in the adolescent rat, particularly with respect to the establishment of dendritic morphologies and their synaptic complement that underlies functional performance. Assessing the mature brain is thus important as animal model data also indicates that exposure to alcohol during development may lead to developmental delay (Wozniak et al., 2004).

1.4 Social Functioning

Hippocampal functioning is essential for every day activities, such as finding one’s way to the next lesson at school, correctly navigating oneself around a large city or remembering important information from a recent lecture. However, there are other, perhaps more important aspects of life which are commonly taken for granted – and which the capacity to uphold may be diminished in children and even adults affected by FASD. The ability to socially interact and ‘play’ with our peers is one important aspect that helps maintain a balanced, enjoyable lifestyle that includes social contact. Social interaction involves a certain level of reciprocity from both parties, and undefined ‘rules’ mitigate the playful interactions between both children and adults alike. Recent studies have suggested these social skills, and inhibitory mechanisms may be altered in children and adolescents affected by some form of FASD (McGee et al., 2008,
Rasmussen et al., 2008, Salmon, 2008). With these relatively new and undocumented aspects of FASD becoming prevalent in the human literature, it is of critical importance to investigate these behavioural anomalies through the use of an animal model. Therefore the primary focus of this thesis will be the investigation of the effects of a single ethanol binge on the structural and functional aspects of social development.

1.4.1 Play fighting in rats

Play behaviour in rats is an important form of affiliative behaviour, which is paramount for the adequate development of social competency (Vanderschuren et al., 1997). The behaviour patterns during play fighting in rats (Rattus norvegicus) were first documented in the literature nearly four decades ago (Poole and Fish, 1975, Pellis and Pellis, 1983), however it was not until the seminal paper by Pellis & Pellis (1987) that the differential aspects of play fighting from serious fighting were analysed.

It was found that in play fighting, the main target of attack (by snout or oral contact) and defence is the nape area of the neck, while in serious fighting the target of attack (usually a bite) is the intruder’s rump or hind limbs. The type of contact imposed in the two types of fighting differed quite considerably. A bite was the usual form of contact in serious fighting, usually leaving a wound, while in play fighting no malicious contact occurs, rather, a soft nudge or contact around the nape area is all that prevails (Pellis and Pellis, 1987).
**Figure 1.4 Sequences of play fighting**

Diagrams depicting rats actively play fighting have been adapted from Pellis and Pellis (1987). These show the attack and defence mechanisms involved in the respective behaviours. During play fighting an attack is initiated via the nape – and can occur from both the front (A) and the rear (B). While the defender is in the supine position, the attacker continues to direct efforts towards the nape (C). The rats depicted in A and B are 31 days old, while the rats in C are 56 days old. The lower case letters represent frames in a sequence of play. Drawn from 16-mm movie film taken at 48 frames/sec.

As depicted in Figure 1.4, the attacking rat can either attack the nape from a direct route by facing the other animal on its hind legs, or by coming in from the flank or rear, and in both instances even after the submissive animal has adopted a supine position the attacker continues to roll over and attack the nape – clearly showing the nape as the primary target in play fighting.
The idea of the nape being the target in play fighting was reinforced in a study by Siviy and Panksepp (1987) that used local anaesthetic to reduce the sensitivity of the nape. This resulted in a decreased frequency in nape attacks on the anaesthetised animal, suggesting that nape contact alone – without defence – is not sufficient for play fighting to be maintained.

Figure 1.5 Multiple attack sequence

A sequence of play fighting in 31 day-old rats represents multiple attempts to attack and defend the nape area. The attacker is targeting the nape from the rear; the defender initially rears up (d), and then takes a supine position (h) while the attacker continues to direct the attack at the nape (l). The sequence is terminated when the defender regains a neutral position, and the defender in this case then became the attacker – displaying the reciprocal nature of play fighting (o). Adapted from Pellis and Pellis (1987).
A reciprocal relationship in the fighting must be present for animals to find reward in the play fighting (Pellis and McKenna, 1995). The prefrontal cortex is involved with analysing and responding to subtle interpersonal cues occurring in play fighting, and which are of great importance to proper social functioning. Playful attack in play fighting consists of one animal placing its snout near the nape, or actively contacting the nape of the partner (Pellis et al., 2006).

Defence during play fighting can involve one of two types of defensive response – either evasive or facing defence. Evasive defence involves moving both the nape and neck away, or running or dodging from the attack. This essentially terminates contact, and this type of defence occurs 20-30% of the time. Facing defence involving moving the head toward the other animal, occurs about 70% of the time, and can be split into 2 categories (Pellis and Pellis, 1987).

1) Turn on axis to face attacker, then rear upwards – this occurs about 10% of the time (Figure 1.5 d).

2) Rotation along the long axis, while forelimbs turn to attack and push with one or both legs, but with at least one hind foot in contact with the ground (partial rotation) (Figure 1.5 e), or animal completely rolls over the long axis of its body to a fully supine rotation with all limbs breaking contact with the floor (complete rotation) (Figure 1.5 i) (Pellis et al., 2006).

Age is a large determining factor regarding the predominant type of defence. During infancy the most common defence is complete rotation, then in puberty the tendency is toward partial rotation, while during adulthood the turn to face defence is most common (Pellis and Pellis, 1990). Sex differences also exist – such as male rats initiating more play-fights than females at all ages, and females retain the supine defensive behaviour rather than the turn to face defence as adults (Pellis and Pellis, 1990).

The rat is an ideal model for play fighting as the rat exhibits similar attributes in play fighting as other mammals in some areas (such as affiliation during physical contact), which putatively depends on the amygdala and cingulate cortex in both rodents and primates (Murphy et al., 1981, Beauregard et al., 1995). As has been seen in the rat, almost all mammalian species show a pubertal decline in play fighting (Pellis and Pellis,
Therefore the rat is a useful and relevant animal model when looking at specific aspects of social behaviour, and normal and abnormal neural substrates can be deciphered through analysis of a specific aspect of play behaviour. For example if an impaired animal has retained the basic innate functions of play fighting, but failed to observe the need for reciprocity during bouts then conclusions can be made regarding which parts of the animals’ brain has been damaged (Siviy and Panksepp, 1987).

Humans also exhibit the need to learn and practise social skills and interactions during childhood and adolescence, so that social understanding and maturity is evident later in life. Playground rough and tumble in children, and the unwritten rules which are essential in maintaining order, are an essential part of learning and mastering these social skills. Play fighting in rats and playground interactions in children are both complex behaviours that depend on the integrity of a number of different cortical and subcortical neural circuits (Euston et al., 2012, Lee and Lee, 2013).

### 1.4.2 Brain areas involved with play fighting

Through various study designs the various cortical and subcortical regions of the rat brain involved with the function of play fighting have been deduced. The cortex has been shown to be important in retaining normal defensive responses in juvenile rats – with neonatal decortication producing animals that behave in a defensive manner similar to adult rats (Pellis et al., 1992, Panksepp et al., 1994). During adolescence rats show a higher proportion of supine defences, while in adulthood this changes to strategies that involve partial and facing defences more frequently. In these studies the decorticated adolescent rats showed a reduced frequency of the supine defence, indicating the cortex is essential for adequate juvenile social interaction and also the generation and modification of defensive strategies later in life. Further to this, the orbital frontal cortex (OFC) has been implicated in the partner-related modulation of defence and in frequency of play bouts observed during adolescence. In this study by Pellis et al (2006), rats with bilateral OFC ablations showed higher levels of play (hyperactivity) and a diminished ability to display submissive behaviour when paired with a dominant playmate.

The medial prefrontal cortex (mPFC) is also intricately involved with play fighting in the rat, and serves to increase the complexity of the defensive response. Rats with
bilateral mPFC ablations display a global shift from a reduction in complete, supine rotations towards more evasive defensive manoeuvres (Bell et al., 2009). Thus it is clear that as the above ablation studies have shown, loss of certain brain areas can lead to very particular changes in social functioning. A population of neurons resident in a specific brain region is always an integral part of a local neuronally based network, which in turn is part of a wider network within the brain. When considering the impact of exposure to ethanol on a specific cell population during development it is essential to understand the position of the neuronal population within the wider brain network. One such region is the hippocampus, which plays a key role in many cognitive functions including a range of memory and learning tasks. It is composed of a number of both hippocampal and parahippocampal regions, each of which has a particular complement of cell types.

1.5 Inputs and Projections to the Hippocampal CA1 area

If considerable cell loss occurs within a structure it is likely that the functional behaviour elicited by that circuit and the structure of other components within the circuit will be altered. The principle cell of the CA1 region is the pyramidal cell, which has a basal dendritic tree extending in the stratum oriens and an apical dendritic tree traversing to the hippocampal fissure across the stratum radiatum into the stratum lacunosum-moleculare – both of which contain specific input pathways (Harris and Stevens, 1989, Shepherd and Harris, 1998). If significant numbers of CA1 pyramidal cells in the hippocampus are permanently lost after a single binge of ethanol, it is reasonable to assume that cortical and subcortical areas intrinsically connected to the hippocampus may also be affected, either as a result of apoptosis due to ethanol exposure or loss of pre or post-synaptic contacts.

1.5.1 Associational / Commissural Connections of CA1

The entorhinal cortex is intricately linked with the hippocampal structure, and sends excitatory glutamatergic fibres to the CA1 region. The CA1 region also has direct axonal inputs back to the entorhinal cortex – creating a reciprocal circuit. The CA1 region contains a large number of interneurons, some of which are located in the
pyramidal cell layer, and although their function is still not yet fully understood, they seem to act as a type of circuit regulator – modifying the excitatory connections via GABAergic suppression (Takacs et al., 2012). The pyramidal cells of CA1 have profuse dendritic branching in the strata radiatum and lacunosum-moleculare layers, with thin axons in the stratum oriens (Davies and Smith, 1980). The major projection from the axons of CA1 pyramidal cells is a topographically arranged projection to the adjacent pyramidal and molecular layer of the subiculum (distal CA1 to proximal subiculum, proximal CA1 to distal subiculum), while the CA1 region also receives a small reciprocal input from the subiculum in a similar manner to that of the entorhinal cortex (Amaral et al., 1991). A weak commissural contralateral projection is present, however this is not nearly as extensive as the commissural projections in CA3 – possibly indicating a distinct functional separation between areas of the hippocampus (van Groen and Wyss, 1990). This may have interesting implications for research in the FASD field, as many of the functional deficits observed are related to the wider hippocampal-limbic pathway – and with the complexity of this circuit still being deciphered, more research is needed to determine the exact pathways damaged by a prenatal ethanol insult.

1.5.2 Extrinsic inputs to CA1

Numerous structures within the brain project to the CA1 region – and are both of cortical and subcortical origin, indicating the fundamental importance of integrity of this structure for normal cognitive and social functioning. The parahippocampal region provides the main source of input in the rat brain, including the entorhinal, perirhinal and postrhinal cortices. Superficial layers of the entorhinal cortex provide the major cortical input to all hippocampal subfields, and this entorhinal input of the perforant (or extra-hippocampal) pathway is located in the stratum lacunosum-moleculare layer of the hippocampus. This is in contrast to the intrahippocampal input from the CA3 region – known as the Schaffer collateral or commissural input, which synapse on the dendrites of pyramidal cells in the stratum radiatum (Takacs et al., 2012). The perirhinal and postrhinal cortices also provide input to the CA1 layer, and together with the pre and para-subiculum serve as the major route through which the anterior thalamic nucleus can affect hippocampal functioning (Pikkarainen et al., 1999).
The anterior thalamic nucleus is just one of the distinct thalamic nuclei that differentially project to hippocampal and parahippocampal structures to relay sensory information and ultimately modulate both processing of sensory stimuli and output. The caudomedial portion of the basal amygdaloid nucleus projects extensively to the stratum oriens and the stratum radiatum of CA1, while the accessory basal nucleus and the posterior cortical nucleus of the amygdala also project to the stratum lacunosum-moleculare of CA1. However the main projections to CA1 are represented predominantly by fibres from the nucleus reuniens, projecting to the stratum lacunosum-moleculare layer of CA1 (and also the subiculum) via the internal capsule and cingulum bundle. Fibres also terminate on thin dendritic shafts – most likely the dendrites of inhibitory gamma-Aminobutyric acid (GABA) interneurons. The projection is topographically organised such that the dorsal portion of the nucleus reuniens projects to more septal parts of CA1 while the ventral reuniens projects more to the temporal CA1. Other inputs include those from the raphe nucleus, nucleus subcoeruleus and the pontogeniculooccipital wave-generating region in the brain stem (Dolleman-Van Der Weel and Witter, 1996, 2000). The distal half of CA1 – adjacent to the subiculum, also receives a significant input from the amygdaloid complex, the primitive fear and emotional centre of the brain and appears to also serve a function in the hippocampal memory circuit (Krettek and Price, 1977).

1.5.3 Efferent Projections from CA1

The extrinsic projections from the pyramidal cell layer of the CA1 region is considerably greater than both the CA2 and CA3 areas, thus the CA1 region may be the critical hippocampal structure in the coordination within the wider brain network. There is a distinct septal to temporal change in the distribution of projections from CA1 pyramidal cells, similar to the topographical map seen in the primary motor cortex. Extrinsic connections are formed from septal levels of CA1, and project to the retrosplenial area of the cingulate cortex (which also has reciprocal connections with the anterior thalamic nuclei). Dorsal portions project to the lateral septum and a nucleus in Broca’s region, along with rostro-lateral parts of the nucleus accumbens. Midseptotemporal levels project to a part of the olfactory bulb called the dorsal peduncular cortex, and also to the pre- and infralimbic cortex. The infralimbic cortex is innervated by temporal levels of the CA1 region, along with the anterior olfactory
nucleus, and anterior and medial parts of the hypothalamus and medial portions of nucleus accumbens. Similar to the afferent fibres from the amygdaloid complex, the CA1 region also sends efferent fibres to the basal amygdaloid nucleus from the temporal two-thirds (van Groen and Wyss, 1990, Takacs et al., 2012).

The medial prefrontal cortex (mPFC) is also a prominent extrinsic target, and in the frontal regions that are innervated by CA1 (mostly prelimbic areas), all layers are innervated diffusely. The ventral prelimbic and infralimbic areas of mPFC are innervated by CA1 and CA2 fields along with the subiculum, and this indicates the robust nature of this limbic circuit – and if apoptotic cell loss were to occur as has been demonstrated in prenatal exposure to ethanol, alarming functional consequences may develop – the magnitude of which are still to be elucidated (Olney, 2004a). The dorsal part of CA1 does not project to the prefrontal cortex, however slightly more caudal and ventral areas show anterograde markers indicating connections in the medial orbital and prelimbic areas, becoming more prominent as tracers are taken more caudal and ventrally in both cortices. Electrophysiological stimulation of the CA1-subiculum region has also been shown to evoke an excitatory response in prefrontal cortical neurons, further confirming this association. Stimulation of CA1 and the subiculum results in long-term potentiation (LTP) in the prelimbic cortex, indicating an important functional link as the prelimbic area is reciprocally connected with the rest of the prefrontal cortex (Jay et al., 1989, Jay and Witter, 1991). Therefore a loss of stimulation from the CA1 area due to cell loss could cause a reduction in the level of LTP in the prelimbic cortex, and subsequent malfunction of the prelimbic-prefrontal cortex circuitry.

Taken simply together, the circuits described above constitute part of the original emotional circuit described by Papez in 1937 (Figure 1.6). By analysing the progress of the rabies virus injected into the hippocampus of a cat, it was possible to identify the various intrinsic and extrinsic connections throughout the limbic system and forebrain (Papez, 1995).
Figure 1.6 The original Papez circuit

This circuit was one of the first representations of how the brain controls emotion, and shows a flow of information from the hippocampus through the fornix to the mammillary bodies, then synapsing with the anterior thalamic nuclei which then innervates the cingulate gyrus, which in turn completes the circuit by synapsing back with the hippocampus. Diagram adapted from Wikimedia commons.

1.6 Cingulate Cortex

Significant acute cell death also occurs in the cingulate cortex after a single ethanol binge, however the long term changes have not yet been assessed. This study (Genge, 2006) found a significant difference in apoptotic cell density in the cingulate cortex (both Acc and retrosplenial) in alcohol treated animals compared with controls, independent of the day of ethanol exposure. The cingulate cortex is part of the wider corticolimbic system – which involves the prefrontal cortex, amygdala and hippocampus (Figure 1.7).
Figure 1.7 The corticolimbic system

Following the doctrine that an extended hippocampal circuit can control emotion, an advanced circuit has been described that also involves the prefrontal cortex, Acc and amygdala – with each structural entity contributing a different emotional or sensory input (Benes, 2010).

Therefore it is important to assess the long-term consequences of an ethanol binge on a single postnatal day, both on the structure and functional output of the cingulate cortex. Recent studies have shown that cingulate cortex-based functions are impaired in alcohol-exposed children and this impairment is not ameliorated with brain maturation (McGee et al., 2008, Rasmussen et al., 2008, Salmon, 2008).

1.7 The role of Anxiety in Social Functioning

It has been widely accepted that acute ethanol consumption results in an anxiolytic, and thus reinforcing effect, serving as one of the main reasons for alcohol dependence and abuse in many people (Conger, 1956, Pohorecky, 1981, Blatt and Takahashi, 1999). The effect of neonatal ethanol exposure on anxiety later in life, is however not so definitive. Chronic prenatal exposure to ethanol has been shown to have an anxiolytic
effect represented by more open arm entries in the elevated plus-maze, and also increase general activity in rats (Gabriel et al., 2006). In contrast to this, chronic ethanol exposure months before Morris Water Maze (MWM) testing has caused an increase in anxiety – like behaviour, represented by wall hugging in the MWM (Santucci et al., 2008). Chronic prenatal ethanol exposure has also produced anxiety-like behaviour, measured as an increase in neophobia in the open field test and decreased open arm entry in the elevated plus-maze (Dursun et al., 2006). Each of these studies used different animal strains, ethanol exposure durations, concentrations and age of testing – hence it is difficult to make solid comparisons. The effect on anxiety of a single ethanol binge during the later period of brain development has not been investigated to date, and findings from such a short exposure may help piece together the varying effects seen in the literature after longer exposures.

Both the elevated plus-maze and the variation of this, the elevated T-maze, have been used as a measure of anxiety in rodents for many years – however there are some subtle differences between them, which can suit one or the other to the particular type of question to be addressed. The elevated plus-maze has been, and still is, a very accurate measure of anxiety in the rodent. However, it is a more generalised test of anxiety – while the elevated T-maze can reveal different types of anxiety-like behaviour, separated into two categories and directly related to clinical conditions. The elevated T-maze is simply the elevated plus maze, with one closed arm blocked off, leaving one enclosed arm and two open arms. Rodents have an innate fear of height and openness, and therefore to be on the open arm (80cm from the ground) is an aversive experience (Montgomery, 1955, Walk et al., 1957, Pellow et al., 1985, Treit et al., 1993). The modern version of the elevated T-maze was derived by Zangrossi and colleagues from the original elevated T-maze described by Viana and colleagues (Viana et al., 1994, Zangrossi and Graeff, 1997).

Unlike the elevated plus-maze, the elevated T-maze can be used to discriminate conditioned from unconditioned responses of fear and anxiety. The maze protocol consists of 2 separate tests, the first being inhibitory avoidance – representing learned or conditioned fear. This is measured as the latency for an animal to leave the enclosed arm and enter one of the open arms – which as a result of exposure prior to testing it has learned to be a stress inducing experience. One-way escape, or unconditioned fear, is measured as the latency to escape from the open arm once placed at the distal end.
Inhibitory avoidance has been linked to Generalized Anxiety Disorder (GAD), while one-way escape is thought to represent behaviour seen in Panic Disorder (PD) (Graeff et al., 1993, Viana et al., 1994, Zangrossi and Graeff, 1997, Graeff et al., 1998).

1.8 Summary and Aims

The impact of alcohol on the unborn foetus during the first trimester of pregnancy has been undisputedly shown to produce severe, enduring physical and cognitive anomalies in both animals and humans. Further research in this field has yielded evidence that alcohol intake during the second and third trimesters of pregnancy also induces deficits, albeit more subtle and variable. Therefore it is important to ascertain what level of alcohol will produce these deficits, and what aspects of structure and function of the developing and mature brain are affected. It is also important to note that FASD is just that – a spectrum of disorders, thus it is critical that continuing research and awareness is possible through both clinical teaching policy decisions at a government level to address this on-going issue in New Zealand.

The aim of this study was to investigate the effects of a single binge exposure to ethanol during the brain growth spurt – and thus model a single binge-drinking episode by a pregnant woman during the third trimester. Investigations focussed on the cellular makeup of important parts of the rat limbic system, and the corresponding social and primitive functioning of these areas during true-to-life situations.
Specific Aims:

1. Investigation of the dose – response nature of long-term changes in brain structure after a single ethanol binge on PN6.
   a. Determine the dose related deficit of pyramidal neurons in the CA1 region of the mature rat hippocampus.
   b. Determine the dose related deficit of neurons in the anterior cingulate cortex of the mature rat forebrain.

2. Use social play as a behavioural measure to assess the function of the prefrontal cortex following a single ethanol binge exposure on PN6.
   a. Determine what dose related changes occur in social play during adolescence (PN32-34) following an ethanol binge exposure on PN6.
   b. Determine what dose related changes occur in social play in the mature rat (PN80-82) following an ethanol binge exposure on PN6.

3. Investigate the dose – response nature of changes in anxiety in the mature rat following a single binge alcohol exposure on PN6.
   a. Determine the dose – response effect of a single binge ethanol exposure on PN6 on inhibitory avoidance.
   b. Determine the dose – response effect of a single binge ethanol exposure on PN6 on one-way escape.

It is hoped that the data generated by the experiments that address the specific aims above, will add to the social-clinical picture of FASD. The ability to become a member of a social grouping is essential in establishing a meaningful life and for many children and adults with FASD this is extremely difficult due to deficits in social skills (Rasmussen et al., 2011). There are many unanswered questions with regard to understanding the aetiology of deficits in social skills and the potential role of intervention programs in ameliorating such deficits for affected children and adults, and it is hoped that this thesis may make some contribution to increasing our knowledge and the life experience of FASD sufferers.
Chapter 2. Methods

2.1 Animals

Timed pregnant female Long Evans rats were obtained from the University of Otago Breeding Unit (Otago, New Zealand). Male and female offspring (a total of n=32 in stereological studies, n=93 in play behaviour study and n=40 in anxiety study) were used in all experiments. The number of animals differed across specific experiments, hence details are given in the methods section for each experiment.

Gestational age, rather than post-parturition age was used to control variations in CNS maturation with respect to birth, as small variations in the timing of birth were present. Pups from timed pregnant dams, where sperm positive day was designated gestational day (GD) zero, were born on approximately gestational day 22 (GD22), hereafter referred to as postnatal day 0 (PN0). Litters were culled to a maximum of 12 pups on PN1 and were kept with the dam throughout the experimental period (except during intubation).

Postnatal days 1-10 in the rat are the equivalent of the human third-trimester, and these animals were exposed to ethanol mid-third trimester, on PN6.

On PN6 pups were marked for identification with a non-toxic permanent marker on their dorsum, and body weight was recorded at the same time of day. Pups were then pseudo-randomly assigned to one of five treatment groups: 6.0 g/kg ethanol (E6), 5.25 g/kg ethanol (E5), 4.5 g/kg ethanol (E4), sham intubation control (IC) or suckle control (SC). Body weight was matched across treatment groups, and no more than 6 pups per litter were assigned to a particular treatment group (within each litter n = 6/treatment/day). This helps to keep the milk supply stable for the alcohol-exposed animals. In order to obtain single sex quadrads for post-weaning housing, cross fostering between litters was sometimes necessary to ensure 8 out of the 12 in one litter were of one sex.
Here is an example of how a typical litter of 12 pups was broken down:

1) M E6  
2) M E6  
3) M SC  
4) M SC  
5) M E4  
6) M E4  
7) M SC  
8) M SC  
9) F E5  
10) F E5  
11) F SC  
12) F SC

Animals were housed under standard conditions of a 12-hour light/dark schedule, with standard rat chow and water available \textit{ad libitum}. On PD25, pups were weaned into groupings of up to 6 same-sex littermates for the cell count study, and groups of 4 (quadrads) for the behavioural studies. All animals were housed under standard conditions, and all animal care and manipulation protocols were approved by the University of Otago Animal Ethics Committee (AEC 10/07) and appropriate conduct was adhered to at all times. Cages were high enough to allow rats to stand on hind legs, and also contained an elevated platform and cardboard pipe for enhanced cognitive stimulation.

A timeline of experiments each individual cohort underwent, along with brief procedural details is displayed in Figure 2.1.
Figure 2.1 Timeline of experiments

This shows the sequence of events following birth on PN0 through to either the stereological studies or the behavioural analysis. All animals, regardless of which group they were assigned, were treated the same until weaning on PN25.
2.2 Intubation procedure

On PN6, pups within litters were pseudo-randomly assigned to treatment groups; three ethanol-exposed and two control groups (a maximum of two littermates per sex, per group). The ethanol-exposure groups were intubated with an ethanol solution (a given volume of 95% ethanol in Intralipid, intravenous infusion, Baxter healthcare Pty Ltd. Fresenius Kabi AB) as a daily ethanol dose of either 4.5 g/kg ethanol (group E4), 5.25 g/kg (group E5), or 6.0 g/kg (group E6). Controls consisted of one group intubated but with no alcohol or ‘Intralipid’ delivered (group IC) and an additional control group of suckle control animals (group SC) comprised the rest of the litter so that no more than 6 animals per litter were alcohol exposed. The alcohol treatment groups received a single ‘binge’ dose of ethanol via intragastric intubation delivered as a specific dose-dependent percent (v/v) solution in an artificial milk solution (Intralipid, intravenous infusion, Baxter Healthcare Pty Ltd. Fresenius Kabi AB). The volume of each ethanol intubation, equivalent to 1/36 of the body weight of the pup, was administered as two intubations of equivalent volumes, two hours apart (Bonthius and West, 1990). Intubation control (IC) animals were given a sham intubation with no vehicle, as milk supplement has been shown to abnormally increase growth rate (Goodlett et al., 1998). Un-intubated or suckle controls were reared normally by the dam, removed with littermates during the intubation procedure, and were used in the behavioural studies but not the stereological study.

All intubations were performed using a custom made cannula formed from a 10cm length of PE-50 polyethylene tubing (Intramedic, Clay Adams, NJ) attached at one end to a 22-gauge needle and at the other to an 8cm length of PE-10 polyethylene tubing (Intramedic, Clay Adams, NJ). The end of the PE-10 tubing was gently flared to round off the cut edges and prevent any scratching as it was inserted down the oesophagus. A 1ml syringe filled with the predetermined amount of intubate, was attached to the needle. The pup was then held by the base of the skull on its dorsum in the palm of the investigator, so the milk spot would become visible and the length of tubing marked to ensure that the appropriate length of tube was inserted. The rat pup was held gently with the body hanging in a vertical position, by placing the thumb and forefinger at the base of the head. The intubation tube was dipped in corn oil to minimise friction and damage to the epithelial lining over the tongue and oesophagus, and gently inserted into the mouth over the back of the tongue, down the oesophagus and into the stomach.
When the tip of the tube was in the correct position the swallowing action of the pup and gentle pressure allowed the tube to pass smoothly down in to the stomach. When the tube was satisfactorily inserted the pup was placed in a horizontal position, ventral surface uppermost on a heated animal pad so that the milk spot could be visualized during delivery of the intubation solution. Each intubation took approximately 15 seconds.

The sham intubation animals had the tube inserted into the stomach in the same manner and it remained in place for 15 seconds but no solution was delivered. During each intubation process (approximately 12 minutes per litter) the entire litter were removed from the dam, to control for separation stress across treatment groups. During the intubation procedure the pups were placed in a small container lined with polar fleece and placed on the heated animal pad. All pups were returned as a group to the home cage on completion of the intubations. Pups were placed at the food hopper end of the cage to allow the dam to collect each pup and return them to the nest area. All pups irrespective to treatment were collected and huddled over in the nest while animals fed. The composition of the litter with 6 control animals insured that inebriated pups received jostling and body warmth from the control animals. After weaning on PN25 all animals were ear marked for individual identification.

2.2.1 E6 treatment group:

Beginning at 9am on the morning of PN6 animals in the 6g/kg (E6) group were given a total of 2 ethanol-containing intubations to constitute the single binge exposure as detailed above. Animals in the E6 group were given two additional feeds of milk, beginning two hours after the second alcohol feeding, on PN6, to ensure their survival and adequate growth, as higher alcohol doses are known to impair suckling in neonatal rats and subsequent nutritional status (Goodlett and Johnson, 1997, Thomas et al., 2004, Gil-Mohapel et al., 2010). The additional milk feeds were the same volume of the intubation delivery but if this did not produce adequate filling of the stomach, as determined by the appearance of the milk spot, the intubation volume was increased by a small amount. This strategy was used in an attempt to equate the additional feedings given to the amount a non-inebriated pup would be consuming during suckling.
2.2.2 E5 treatment group:

All procedures were carried out as for the E6 group. The volume of alcohol delivered was the same volume per body weight but the 5.25g/kg solution in milk substitute contained a lesser v/v concentration of ethanol. Thus not only the daily doses of ethanol, but also the concentration in the stomach will have contributed to the different peak blood ethanol concentration per dose. This is due to the concentration of alcohol delivered altering the initial absorption of alcohol from the stomach prior to absorption in the small intestine. These animals also received two top-up feeds of milk as detailed above to compensate for the lack of suckling following ethanol intubation.

2.2.3 E4 treatment group:

All procedures were carried out as above for the E5 and E6 group. The 4.5g/kg ethanol milk substitute solution delivered contained a lower concentration of ethanol but was based on the same volume per gram of body weight. The E4 group also did not receive any top up milk feedings due to the relatively low dose of alcohol and their ability to continue to suckle during and after the alcohol intubations as ascertained by the continual appearance of a milk spot of equivalent size to that of the control animals. These animals received a sham intubation twice at two hourly intervals after the alcohol intubations to match for the stress of the additional intubations given to the E5 and E6 animals.

2.2.4 Sham intubation control (IC) group:

These animals were treated exactly the same as all alcohol exposed groups, except no vehicle solution (Intralipid, intravenous infusion, Baxter Healthcare Pty Ltd. Fresenius Kabi AB) was given during intubations. The intubation tube was inserted into the stomach, and was held in place for a period of 15 seconds (the average time for an intubation). Two sham intubations were given to control for stress. No milk solution was administered to intubation control animals as it results in excessive weight gain compared to both suckle controls and alcohol exposed animals (Goodlett and Eilers, 1997, Goodlett and Johnson, 1997, Light et al., 1998).
2.2.5 Suckle control (SC) group:

These pups did not receive any intubation or blood ethanol concentration (BEC) procedures. The only manipulation the suckle animals received was removal from the dam as part of the litter during the intubation procedures and all weighing and marking procedures that were carried out on IC and E group pups.

2.3 Blood Ethanol Concentration (BEC)

Using the tail clip method, a 20µl blood sample was taken from all ethanol-intubated and IC animals, 90 minutes after the last ethanol delivery – as this has been shown to represent peak blood ethanol concentration ((Kelly et al., 1987, Loughnan, 2001) and (Napper lab unpublished data). A sterile stainless steel surgical scalpel was used to cut off the most extreme tip of the pup’s tail. Blood was then evacuated by gently sliding 2 fingers down the tail, and was collected in a 20µl heparinised (to stop clotting) capillary tube from both the alcohol exposed and intubation control pups. Each sample was then piped into a small glass screw top vial that contained 180µl of internal standard plus a preservative solution. Blood ethanol concentration was determined using gas-chromatography (ESR Forensic, Upper Hutt, NZ).

2.4 Paw Marking

On PN8 animals were paw marked for long-term identification. A 27 gauge sterile needle was attached to a 1ml syringe and a very small amount (< 0.02ml) of black Indian ink was injected into the subcutaneous fat pad on the underside of the paw. Only enough ink was injected to leave a small black circle under the skin. The paw (s) was then wiped clean before the pup was placed back into the home cage. This system of marking allows up to 15 individual marks per litter. A diagram of the paw marking schedule is illustrated in figure 2.2.
Figure 2.2 Paw marking placement

Each number was not actually tattooed on the paw, rather a small dot, which represented either 1, 2, 4, or 8 allowing a single number or a combination of numbers up to 15.

2.5 Tissue Collection

2.5.1 Perfusions

On PD 365 animals were deeply anesthetised (sodium pentobarbitone (60 mg/kg i.p.) (Pentobarb 300, National Veterinary Supplies Ltd., Auckland, New Zealand) and transcardially perfused with heparinized phosphate buffered saline (1 x PBS, pH 7.2), followed by 4% paraformaldehyde in 1 x PBS.

2.5.2 Removal of brains

Immediately following perfusion, brains were carefully removed from the skull cavity. Tissue scissors were used to dissect away the layers of dermis, muscle and supporting structures around the mid neck area – leaving the upper spinal cord exposed. Heavy-duty scissors were then used to transect the spinal cord. Precision bone cutting forceps were used to carefully cut away the bony casing of the spinal cord and skull, leaving the olfactory bulbs, forebrain, cerebellum and a portion of the brainstem intact. A small spatula was inserted under the base of the brain and spinal cord and gently manoeuvred to sever the cranial nerves and connective tissue. Brains were then placed into small vials containing 4% paraformaldehyde fixative solution and refrigerated at 4° Celsius.
Two days later the fixative solution was replaced with a 30% sucrose solution (in 1x PBS), and were not removed for further analysis until the brains had sunk, indicating the sucrose had completely infiltrated the brain.

2.5.3 Tissue sectioning

Prior to immersion in sucrose-PBS solution, brains were cut into forebrain, cerebellum and brainstem components using a disposable razor blade. The forebrain and cerebellum were separated by sectioning along a coronal plane down the cerebral–cerebellar fissure. The brainstem was separated from the cerebellum on a transverse plane by cutting through the cerebellar peduncles. The distal end of the brainstem was defined as the point where the fibre tracts finished tapering down from the medulla to the narrower spinal cord and was severed from the spinal cord at this point. Excess fluid was removed and each portion was placed on lint-free weighing paper, weighed on precision scales and immediately placed into a 30% sucrose-PBS solution to prevent dehydration.

2.5.4 Tissue preservation

Pellets of solid carbon dioxide were crushed into a fine powder and placed into a polystyrene container. Each forebrain, cerebellum and brainstem section was coated in a thin layer of the mounting medium Tissue-Tek® (O.T.C™ Compound, Sakura Finetek, Europe), then covered in dry ice for 2 minutes. The 3 brain portions from each animal were then retrieved using a small plastic spoon and wrapped in aluminium foil and placed into a small, labelled zip-lock bag. A specific code was then assigned to each animal from a random number list of 0-100, to allow all stereological and other analysis to be carried out by an investigator blind to treatment group. The brain tissue was then frozen at -80 degrees Celsius to preserve for further analysis.

2.5.5 Cryostat sectioning procedure

Before cutting the forebrain on the cryostat 2 brains were transferred at a time into a -20 degree Celsius freezer for 24 hours. This reduced the physical hardness of the frozen tissue and achieved superior sections. Each forebrain was mounted on the cryostat mounting plate for cutting in the coronal plane using Tissue-Tek®. The forebrain was cut throughout the entire length (minus the olfactory bulbs) at a thickness of 60µm using a steel non-disposable cryostat knife. For every 4 sections cut two were placed in
order into wells of a 12-well plate filled with cryoprotectant, one was then discarded, and the final slice placed into a well containing 1 x phosphate buffer solution (PBS). After sectioning the PBS slices were systematically mounted onto standard double gelatine subbed glass slides and labelled with the specific animal code. The sections stored in the cryoprotectant were covered with parafilm then placed into a -20 degrees Celsius freezer for preservation. Some of these were used in the Neu-N immunohistochemical study, and the remainder were stored for possible future studies.

2.6 Stereological investigation using the optical disector

2.6.1 Neu-N immunohistochemistry

Before stereological analysis could be carried out it was important to establish criteria to ensure consistency in identifying the nuclei of the CA1 pyramidal cells over other neurons, glia and endothelial cells that are present in the CA1 region. To achieve this, a sample of brain slices from control animals were labelled with an antibody to Neu-N, a neuronal specific marker, using immunohistochemical procedures.

2.6.1.1 Neu-N Immunohistochemistry protocol

All steps were carried out at room temperature unless stated otherwise, and sections were washed in phosphate buffered saline between each step. All processing was done on free floating sections in wells, and solutions withdrawn carefully with a pipette. All processing was done on a slow moving shaker table.

Day 1: Slices were rinsed in 1 x PBS for 20 min then aldehyde residue was removed by quenching in 0.1M glycine for 20 min, followed by 2 x 20 min 1 x PBS washes. A block (100μL HIGS in 900μL 1% PBS/BSA (Bovine Serum Albumin)) was then applied for 1 hour. The blocking solution was removed from sections, and the monoclonal primary antibody; NeuN (diluted 1:200 in 1% PBS/BSA) (Abcam, ab13938, monoclonal) was applied for 48 hours at 4°C.

Day 3: Primary antibody was removed and discarded, and sections were rinsed with PBS. Slices were washed with 1xPBS 3 times with each wash lasting 1 hour. Blocking solution (100μL HIGS in 900μL of 1% PBS/BSA) was applied for 3 hours at room temperature. The block was then removed and the sections were incubated in secondary
antibody; biotinylated goat anti-mouse (diluted at 1:200 in 1% PBS/BSA – pH 7.4) (Amersham, RPN1177) overnight at 4°C.

Day 4: Secondary antibody was removed and sections were washed 3 times in 1xPBS with wash duration of 1 hour. Slices were then washed in 0.5% hydrogen peroxide (H₂O₂) in methanol (3.33ml 30% H₂O₂ adding in 200ml methanol) for 15 min. One ten min 1xPBS rinse followed by 2 x 30 min rinses in 1xPBS were carried out to remove all methanol from the sections. Slices were then incubated in biotinylated Streptavidin-HRP (Horse Radish Peroxidase) (Sigma, A3682) (diluted 1:100 in 1xPBS) followed by three 30-minute washes in 1xPBS.

Visualization: Sections were visualized using a Nova Red stain vector kit for peroxidases (SK-4800). 100μL of Nova Red solution was added to each well containing a section, and incubated for exactly 15 min. The reaction was stopped by sucking out the solution and washing with milli-Q water for 3 x 1 min.

2.6.1.2 Mounting: Dehydration and mounting in Xylene

Sections were mounted onto gel-coated slides. Slides were dipped in Harris Haematoxylin 3 times, into a water wash for 3 minutes, a 6 second wash in 95% acid alcohol and a final 3 minute wash in water. Slides were then dehydrated through a graded series of ethanol (75% ethanol, 6 sec; 95% ethanol, 8 sec; 100% ethanol, 8 sec) then finally 2 rinses of xylene for 1 min each. Sections were cover slipped with DPX (Distrene, Plasticiser, Xylene) and allowed to dry in the dark. All sections were viewed using a light microscope (Olympus AX70) with a digital camera interfaced to a computer.

2.6.2 Light microscopy for neuronal identification

The control sections were analysed to establish a set of criteria that could be used to identify the cell nuclei of pyramidal neurons in Nissl stained sections. As can be seen in the photomicrograph (Figure 2.3), the cell bodies of pyramidal neurons are much more circular and larger than the glial cell bodies, which were stained a light purple colour. The nuclei of all types of neuron were more prominent and were surrounded by considerably more cytoplasm than glial and endothelial cells. Clumped chromatin, seen as densely stained irregular shaped bodies, was usually prominent in the nuclei of the
neurons. Cell processes could be seen on the neurons as the focal plane was moved through the depth of the section and were much more prominent in neurons than in glial cells. The pyramidal neurons typically displayed a distinct apical dendrite that was visible in through a number of focal planes and several basal dendrites also leaving the cell soma. The thionin stain used in the stereology identifies Nissl substance (rough endoplasmic reticulum, RER), and this is present in the initial segment of the broad dendritic processes as they leave the soma, allowing them to be visualized. Astrocytic processes are very fine as they leave the region of the cell body and as they contain only a small amount of RER do not bind much stain, meaning only the astrocyte cell body, with dominant nucleus is visible in the light microscope.

Endothelial cells were easily identified by the granular appearance of the nucleus, and could have a shape ranging from sickle-shaped to spherical depending on the plane of the section (Figure 2.3). Endothelial cell nuclei were also much smaller than those of neurons and glial cell nuclei. To verify the identification of an endothelial cell the focal plane could be moved through the depth of the section to follow the lumen of a blood vessel, which had a very clear appearance of the endothelial cells lining the walls. As for the glial cells, the endothelial cell nuclei were stained a light blue colour, a result of the differential staining with Harris Haematoxylin (Figure 2.3).

Most glia present were putatively astrocytes – and although these cells typically have numerous cell processes these were not present as the Neu-N immuno protocol only visualizes the Neu-N protein only present in neurons. The thionin stain used in the stereology identifies nissl substance (rough endoplasmic reticulum, RER), and this is present in the initial segment of most neuronal processes, allowing them to be visualized – while this is not present in astrocytic processes.
Figure 2.3 Photomicrograph showing CA1 neurons positive for Neu-N

The dark red/brown cells are CA1 pyramidal neurons of the hippocampus (PC = pyramidal cell in focus, PC₁ = pyramidal cell out of focus. Sickle – shaped, dark blue/purple cells are endothelial cells lining blood vessel walls (E = endothelial cell in focus, E₁ = endothelial cell out of focus). Light blue/purple, spherical shaped cells are most likely glial astrocytes (G = glial cell in focus, G₁ = glial cell out of focus). Photomicrograph taken at 100X oil immersion lens on Olympus AX-70 microscope.
2.6.3 Thionin Staining protocol

Sections to be analysed using stereological methods (see section 2.5.5) were cut on the cryostat and float mounted from PBS onto slides. Sections were placed in slide holders and fan-dried for approximately 12 hours to remove all moisture and ensure the sections were firmly adhered to the slides prior to staining. In order to stain cell nuclei through the entire 60µm thickness of the section, a dilute (0.03%) thionin solution was used. The procedure used was as follows; slides in a slide rack holding 20 slides placed into distilled water for 2 minutes to rehydrate the tissue sections and were then immersed in a 0.03% thionin solution for 25 minutes. Following this, sections were immersed in distilled water for two washes of 1 minute each. Sections were then dehydrated through a series of alcohol of increasing concentrations, 50%, 75% and 95% for one minute in each. Slides were then placed in 95% acid alcohol solution (200ml 95% ethanol and 10 drops 100% glacial acetic acid) for 25 minutes followed by two rinses in 100% ethanol, each of 1-minute duration. Sections were then placed in 100% xylene, and cover slipped using the mounting medium DPX. The cover slipped slides were inverted and slight pressure applied to the slide to ensure that there was no extra mounting medium over the section that could interfere when focussing through the thick section using a 100X oil immersion objective lens. Slides were covered to exclude light and placed into a fume hood to dry for 12 hours. This procedure ensured pyramidal cells, glia and endothelial cells were all easily identifiable (Figure 2.4).
Figure 2.4 Photomicrograph of thionin-stained section of CA1

This photomicrograph is representative of what was visualized when undertaking all stereological procedures. The triangular/spherical shaped dark blue/purple cells are CA1 pyramidal neurons of the hippocampus (PC = pyramidal cell in focus, PC₁ = pyramidal cell out of focus). Sickle – shaped, light blue cells are endothelial cells lining blood vessel walls (E = endothelial cell in focus, E₁ = endothelial cell out of focus). Light blue, granular and spherical shaped cells are most likely glial astrocytes (G = glial cell in focus, G₁ = glial cell out of focus). Photomicrograph taken at 100X oil immersion lens on Olympus AX-70 microscope.
2.6.4 Delineation of brain regions for stereological analysis

2.6.4.1 Delineation of CA1 region

CA1 delineation was determined primarily through the extensive description by (West et al., 1991). Reference to both the latest rat brain atlas (Paxinos and Watson, 2005), and literature which used gene expression to map out the various regions of the rodent hippocampus (Lein et al., 2007, Thompson et al., 2008) was also used for clarification. The delineation of the subiculum from CA1 was defined as where the more dispersed cells of the subicular layer were more closely packed and tapered to the narrower layer of densely packed pyramidal cells of CA1 (Figure 2.5). The delineation of the CA1 sub-region from the CA2 and CA3 sub region was not always clear but a distinct change did occur at the lateral end of the CA1 region. For this reason the boundary was referred to as the boundary between CA1 and the CA2/CA3 sub region. This was defined as the point where the tightly packed pyramidal cells of CA1 became less densely packed and the layer was noticeably wider (Figure 2.6). The pyramidal cells of CA2/CA3 were more dispersed and the pyramidal cell bodies increased in diameter, however the CA2 area was not always present or easily identifiable and in this case the delineation was marked at the CA3 border which is much more distinct. In more caudal sections the area of CA1 was very distinct and separate from CA2 and CA3 areas. As the pyramidal cell layer became less compact – these sections were delineated by where the dark band of CA1 began and finished. Prior to the initial counting two sets of sections, one from a control animal and one from an E6 animal, blinded to experimenter, were used to verify that the CA1 delineation was constant across treatment groups and could be made repeatedly in a reliable manner on all sections.
Figure 2.5 Delineation of CA1 / subicular region in the rat hippocampus

As discussed above, the CA1 region was determined to begin where the sparsely packed cells of the relatively wide subicular layer tapered off to the large, densely packed cells of the narrow CA1 area. The region of the dentate gyrus (DG) is also depicted. Photomicrograph taken on a 4X lens on the Olympus Ax-70 microscope.
**Figure 2.6** Delineation of the CA1 / CA2/3 region in the rat hippocampus

The CA1 area terminated where the densely packed cells gave way to the more loosely packed and larger, wider cells of the CA2/3 layer. The region of the dentate gyrus is also shown for reference (DG). This photomicrograph was taken with a 4X lens on the Olympus AX-70 microscope.
2.6.4.2 Delineation of Anterior Cingulate Cortex region

The anterior cingulate cortex (Acc) is located in the medial prefrontal cortex (mPFC) and sub serves a range of executive, cognitive and emotional functions through a diverse range of interconnections with the hippocampus, dorsolateral thalamus, amygdala, hypothalamus and brainstem nuclei. It can be separated both structurally and functionally into two distinct regions – the anterior cingulate cortex dorsal (Acd), and the anterior cingulate cortex ventral (Acv).

For the purpose of this investigation, the Acd and Acv were grouped together as the demarcation between the two areas was not possible at 4X magnification.

The most rostral aspect of the Acc coincides with the appearance of the forceps minor and claustrum (Paxinos and Watson, 2005), while the most caudal aspect ends and becomes the retrosplenial cortex (Rsc) where the CA3 region of the hippocampus first appears (Jones et al., 2005, Paxinos and Watson, 2005).

Demarcation of the boundary between the Acd, the secondary motor cortex and the prelimbic cortex was extremely difficult to identify and therefore the most rostral portion of the Acc (which coincides with the Acd) was not included in the delineation of the Acc (Figure 2.7). Thus, delineation began at the appearance of the genu of the corpus callosum (which coincides with the transition between the prelimbic cortex and the Acv).

Medially the Acc was delineated from the intersection of the corpus callosum with the midline, then laterally following the corpus callosum and cingulum until it reached the most dorsolateral aspect. From this point, the Acc was connected to the most dorsomedial aspect of the cortex (Wolf et al., 2002).
**Figure 2.7** Areas in the cingulate cortex

A dorsal view of the rat brain showing the cingulate cortex is medial and extends only slightly laterally (A). A medial sagittal view of the cingulate cortex showing area subdivisions (B). Cerebellum and brainstem have been removed. Adapted from (Jones et al., 2005).
Figure 2.8 Boundaries and cellular morphology of the Acc region

Photomicrograph showing the boundaries of the Acc within which the total number of neurons was determined. Shaded area represents counting frame delineation area. Magnified inset photo represents the morphology of the various cell types in the Acc. N = neuron (in focus), N1 = neuron (out of focus under glial cell), G = glial cell (in focus), G1 = Glial cell on top of neuron, E = endothelial cell of blood vessel.
2.6.5 Stereological analysis

All counting of neurons was undertaken using stereological methods and was performed with the experimenter blind to the treatment group and unique animal codes were not revealed until all counting procedures were complete.

All stereology was undertaken using a microscope (Olympus, BH2) with attached video camera (Panasonic F15) interfaced to a computer with the stereology software package (Cast Grid, Olympus). A microcator (Heidenhain) was attached to the microscope to measure movement of the stage in the Z-axis. Delineations of the hippocampal CA1 area and Acc as described above were applied consistently to all sections.

2.6.5.1 Optical dissector settings for CA1

The primary delineation of the hippocampal CA1 area was marked, using Cast Grid software, on the image of the section as seen on the computer screen using the 4X objective lens. The x100 oil immersion objective lens was used for the optical dissector method and the set of systematic random sampling points was applied within the delineated boundary using the Cast Grid software. The sampling points were 100µm apart in both the X and Y direction within the sampling grid and at each point, an unbiased sampling frame with an area of 259µm² and a depth in the Z axis of 10µm was applied. This constituted the sampling cube within which the pyramidal cells were counted according to conventional stereological unbiased counting rules. Preliminary data had shown that this sampling grid and sampling cube size gave reproducible count data in repeated counts across a number of sections and adequate cell counts across the entire CA1 region when repeated within a known control and alcohol-treated brain. For each optical dissector sample a guard zone was used to exclude any abnormal tissue that may have resulted from damage to the uppermost surface of the section during cutting – this was 2µm from the top of each section (as measured from where the first cells were clearly in focus). A lower guard zone of 2µm or greater, at the lower surface of the section, was used and was determined by the inclusion of all tissue below the 10µm Z-axis depth of the counting cube.
2.6.5.2 Optical disector settings for Acc

All settings were the same as above for the hippocampal CA1 area apart from the X and Y step length, and the counting frame area. The sampling points used were 260μm in both the X and Y direction, and a new sampling frame area of 518μm$^2$ was used to accommodate the larger delineation area for the Acc.

2.6.5.3 Stereological counting procedure

Cell counts were obtained from a random, systematic, unbiased sample through the entire extent of the CA1 region of the hippocampus and the Acc. A random systematic sample of every 3rd section, with random application of sampling frames within each section, was used to determine neuron number with the optical fractionator method (West et al., 1991). Briefly, an unbiased counting frame was placed within the boundaries of the CA1 or Acc region in a random systematic pattern and the optical disector method was used to count the number of neurons at each sampling location. The total number (N) of CA1 pyramidal neurons and Acc neurons was estimated by:

$$N = \sum Q^- \left( \frac{t}{h} \right) \left( \frac{1}{asf} \right) \left( \frac{1}{ssf} \right)$$

Here $\sum Q^-$ is the total number of neurons counted falling within the boundaries of the CA1 or Acc region. The section sampling fraction, $t/h$, represents the height of the disector (h) relative to the measured section thickness (t). The areal sampling fraction, asf, represents the area of the sampling frame as a fraction of the area of the sampling step. The section sampling fraction, ssf, represents the fraction of sections sampled from through the entire length of the CA1 or Acc region.

2.6.6 Statistical analysis

All statistical analysis was carried out using Graph Pad Prism version 5.0c software (Graph Pad Software Inc.). One way ANOVA was carried out on these data, and where a post-hoc test was appropriate the Newman-Keuls test was used.
2.7 Play Behaviour

2.7.1 Housing

Following treatment on PN6 as described earlier, litters were placed back into the original cage with their respective dams and placed back in the animal housing unit.

On PN25 the animals were weaned and assigned to a single sex quadrad containing 2 alcohol treated animals from the same alcohol treatment group and 2 suckle control (SC) animals. This housing arrangement was configured with both alcohol and control animals together as it was important to simulate a real world situation as closely as possible. Housing only alcohol animals together may have resulted in further social decline due lack of normal interaction. All animals were housed in standard plastic and wire rat cages. On the same day as weaning the quadrads were transported to an alternative facility (The University of Otago Taieri animal breeding facility) where play behaviour and all further studies were carried out. The animals were placed on a reverse light cycle, lights off at 7am and lights on at 7pm and allowed to habituate to the change in environmental conditions prior to play behaviour on PN32.

2.7.2 Play chamber and camera specifications

The play chamber consisted of a 50 x 25cm Perspex box with the rear wall formed by a mirror to allow both a front and rear view of the animals in the box to be seen which aids in the analysis of animal interactions. Standard wood chips were placed in the bottom of the box to absorb excrement and were replaced after each day of testing. Despite the camera having a ‘Night Shot’ facility to allow filming in the dark the field of infrared illumination was not sufficient to illuminate clearly the full width of the play chamber and three red lights (40 Watt bulbs) were placed at strategic positions around the room to illuminate the testing chamber for filming. One was placed directly above the testing chamber, and one above and below the camera, at the same 45° angle the recording camera was facing. Together, this lighting system provided a clear view of the testing chamber and an optimized video image, without creating light visible to the animals. A Sony DCR-HC38E digital Handycam (40X optical zoom, Carl Zeiss Vario-Tessar lens) was used to record the play fighting sessions. Sony mini digital video 60-minute tapes, set up in short – play mode for higher quality pictures of 30 frames per
second were used for all recording. The camera was set up on a tripod in the testing room, with another identical camera placed next to the recording camera to provide extra infra-red illumination of the chamber. All videos tapes were uploaded via DV cable onto a Western Digital 1TB dual-disk hard drive via the i-Movie application. Videos were then viewed using QuickTime media player for mac.

2.7.3 Recording protocol

On PN29 each quadrad was placed into the testing chamber for a period of 30 minutes to habituate and familiarise them to the chamber. This was repeated for 3 consecutive days. At the end of habituation on PN31 all animals were placed into individual cages and isolated for 24 hours to increase their tendency to play. On PN32 play fighting between pairs of rats from each quadrad was video recorded for a period of 20 minutes a day for 3 consecutive days. With in each quadrad, each animal played with each other animal once. At the end of each 20-minute play session the 2 animals were placed back into one of the single isolation cages together for 40 minutes. They were then separated and placed back into their individual isolation cages for 24 hours in preparation for the next day of testing. At the end of the testing period animals were placed back in their original quadrads until further testing on PN80.

Beginning on PN77 each quadrad was habituated in the play behaviour chamber for 30 minutes, for 3 consecutive days as previously described for the adolescent animals. On PN80 – PN82 animals were tested again, using the same protocol as described above. For the PN77 animals, the floor area of the play chamber was increased by moving the rear wall back 20cm to adjust for the increase in animal size. After testing animals were kept in their quadrads under standard group housing conditions. At around one year of age animals were transferred to large floor cages housing 10-12 animals. All cages contained animals from each treatment group.

2.7.4 Scoring Criteria

Although a number of software packages have been developed in recent years to automate the analysis of animal behavioural data (e.g. Clever Sys Inc.) the complexity of the interactions that occur between a pair of rats during adolescent play fighting and the speed at which the behaviour occurs necessitate that the analysis is carried out
manually via frame by frame video analysis. Analysis of the interaction also requires considerable precision as the approach of one rat to another can only be classified as an attack if the nose of the attacking animal reaches within a very defined area, the nape region of the neck, rather than a general approach to the dorsum of the body. Currently, a software system with these highly refined capabilities is not available – however this field is growing rapidly and it will be of interest to see how software in this area is being developed with the behavioural phenotyping researcher in mind over the next few years.

In order to transform these complex interactions into quantitative data for statistical analysis a scoring protocol similar to that of Pellis & Pellis (1987) was adopted. The first scoring act is always the attack initiated by one animal towards another and this is scored as one point. In response to being attacked, the defender can do one of three things: evade, face the attacker, or not respond. An evasive defence was scored if the defender moved both the nape and neck away from the attacker; ran away from the attacker or dodged the attack – essentially terminating further contact. This evasive type of defence in rats typically happens in response to around 30% of initiated attacks. The ‘facing defence’ category consisted of either a partial or supine defensive strategy. A ‘partial defence’ was scored if the defending animal rotated along the long axis of its body, and pushed the attacking animal with the forelimbs and one or both legs, but with at least one foot with its plantar surface in contact with the ground at any time. A partial defence was also scored if the defender reared upwards to face the attacker, or placed the front or back paws up in a defensive position – “boxing”. A ‘supine defence’ was scored if the defending animal completely rolled over the long axis of its body to a fully supine rotation with all limbs breaking contact with the floor. Facing defence in rats typically occurs in response to around 70% of attacks. The third type of defence is when the defender does not respond to an attack at all. The defender typically ignores the attack and either sits still in one position or continues grooming, digging the substrate or whatever activity is was engaged in prior to the attack. This type of defence only occurs in response to 1-2% of all attacks.
2.7.5 Statistical analysis

Statistical analysis of the play behaviour section was performed using Graph Pad Prism version 5.0c (Graph Pad Software Inc.) was used for one-way and two-way ANOVAs and descriptive statistics (computer software supplied by the Department of Anatomy and Structural Biology, University of Otago). Post-hoc analysis using the Newman-Keuls adjustment was also applied in some situations. Data were also analysed using a linear mixed model approach, with treatment, number of attacks, and type of defence as variables. Stata 11 (Stata corp. LP 2011) statistical package, in consultation with Dr Peter Herbison, Statistician, Department of Preventative and Social Medicine, University of Otago. This linear mixed model approach ensured that individual variances were also taken into account, as conventional statistical methods such as the ANOVA only analyse group variability. Data from both male and female animals within each group were pooled for statistical purposes, as ethical and financial restrictions on animal number prevented larger group sizes, and hence would have yielded low statistical power if sexes were divided.

2.8 Elevated T maze

2.8.1 Animals, Housing and Apparatus

A total of n=40 rats aged from 12 and 18 months old with age balanced across treatment groups, were tested using an elevated T-maze paradigm based on that of Zangrossi and Graeff (1997). The T-maze procedure was carried out during the dark phase of the animal’s day with animals carried to the testing room in a standard rat cage, covered by a dark cloth to simulate a moonlight night. On the initial exposure day 2 animals were present in the testing room at any one time after which only one animal was present at any one time.

The elevated T-maze was converted from an elevated plus-maze by blocking off one of the 2 enclosed arms with a piece of white Plexiglas, leaving 2 open arms intersected by a closed arm. (see figure 2.9) The maze was constructed of medium density fibreboard, and was elevated 80cm off the ground by a black anodised steel frame. Each arm was 50cm in length and 13cm wide, with a 15cm by 15cm intersection in the centre. The two open arms had 2cm high surrounds to prevent animals from falling off the maze. The walls surrounding the enclosed arm were 18cm high, which was sufficient to allow
animals to rear while preventing escape over the top. The wooden portion of the maze was mounted and secured to the steel frame using Velcro, then the frame secured to the floor using standard duct-tape to prevent movement from knocks. The maze was centred in the testing room, with the video camera (Panasonic WV-CP284CH) mounted on the ceiling directly above the centre of the maze. The computer was set up on a table in the testing room to allow ease of data entry. Two 40Watt lamps were set up elevated, on opposite sides of the open arms to create enough light for the animals to be aware of their surroundings, but with enough ambience to keep stress levels to a minimum after coming from a reverse – light cycle room. The video camera used was a Panasonic WV-CP284CH feeding images back to a Dell computer running a software package, Clever Systems Topscan. The elapsed time to escape back into the closed arm, or time to leave the closed arm with all 4 paws was obtained through manual clicks of a wireless mouse on the Topscan program. Data was exported to Microsoft Excel 2011, then Graphpad Prism version 5.0c (Graph Pad Software Inc.) used for statistical analysis. One-way ANOVA was used to assess the effect of treatment, while two-way ANOVA was used to assess the interaction of treatment and trial number on latency. Where necessary, post-hoc analysis using the Newman-Keuls adjustment was used to identify individual group differences.
Figure 2.9 Elevated T-maze apparatus

As discussed the elevated T-maze was converted from an elevated plus-maze by placing a barrier at the end of one closed arm. Maze is set up for inhibitory avoidance and one-way escape.
2.8.2 Elevated T-maze Procedure

2.8.2.1 Exposure trial

The elevated T-maze protocol for all trials matched that of Zangrossi and Graeff (1997). Preceding each session with a new animal the maze was wiped down with a solution of disinfectant (Hibitane) in 95% alcohol to remove any odour remaining from the previous animal that may introduce bias in the animal’s performance. A white Plexiglas barrier was placed at the centrally located end of each open arm to force the animal to remain in the open arm. The exposure trial was used to habituate the animal to the open arm, subsequently reducing the time to escape during the one-way escape trials as the innate reaction to a novel environment is nullified (Zangrossi and Graeff, 1997).

Two animals at a time were exposed concurrently in the two open arms of the maze – one animal in the left arm and one in the right, with the barrier in between. The animals were assigned at random with treatment group counterbalanced between arms. The animals were placed at the most extreme end of the open arms and were recorded for 30 minutes. If an animal jumped off the platform it was placed back on at the point of original placement at the end of the arm. The exposure trial was only completed once per animal and on the day prior to avoidance and one-way escape testing.

2.8.2.2 Inhibitory Avoidance Testing

The animal was placed in a plastic container filled with wood chips for two minutes prior to the first avoidance test (baseline). The animal was then picked up with two hands and placed into the distal end of the closed arm with one hand around the shoulders, with its head facing the centre of the maze. Directly after release of the hand a wireless optical mouse was used to start the timing within the Clever Systems software. The time was stopped when the animal walked with all four paws over a line that delineated the end of the closed arm. The animal did not have to enter another arm; it only had to leave the previous arm for the trial to be completed. A maximum of 300s was allowed for the animal to exit the closed arm, and if this was reached the animal was removed and placed back in the container. After completion the animal was placed back in the container for 30s before the next trial. This exact procedure was completed 2 more times for each animal (avoidance 1 and 2).

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2.8.2.3 One-way escape testing

After completion of the 3rd avoidance trial the animal was placed back in the container for another 30s before commencing escape testing. For escape testing the animal was placed at the extreme end of the open arm that had been used for the exposure test, with the head facing the centre of the maze. As for avoidance testing, the trial commenced upon release of the hand from the animal. The baseline escape was recorded as the time taken to leave the open arm with all four paws over the line that marked the delineation between the end of the arm and the central square area. The animal did not have to enter another arm. Again, if not completed within 300s the animal was removed and placed back in the holding container. After 30s in the holding container this exact procedure was completed 2 more times (escape 1 and 2). After the 3rd trial the animal was returned to the home cage in a different room and the next animal brought in for testing.
Chapter 3. Hippocampal CA1 Stereological Investigation

3.1 Introduction

It has become widely accepted that postnatal ethanol exposure during the third trimester equivalent in the rat, producing BECs higher than 200mg/dl, results in acute cell death in various brain regions including the cerebellum, olfactory bulb and hippocampus (Bonthius and West, 1991, Pauli et al., 1995a, Goodlett and Eilers, 1997, Goodlett et al., 1998, Genge, 2006, González-Burgos et al., 2006, Gil-Mohapel et al., 2010).

Such acute neuronal loss has subsequently been shown to result in permanent cellular deficits in these brain regions, which may impact on the correct function of a specific area. Purkinje cells in the cerebellum are one neuronal population known to be particularly susceptible to ethanol toxicity, and after a single ethanol binge there is a dose – response depletion in these cells in the cerebellum of adolescent rats (Goodlett and Eilers, 1997). The method of administration in the aforementioned study was via artificial rearing – which typically produces lower peak BEC values (Napper and West, 1995), however the very high ethanol concentration of 6.6g/kg may have attenuated this factor. This extreme vulnerability and subsequent Purkinje cell deficit occurred after a single binge on PN4, but not for a binge on PN9 – indicating a small temporal window in this cerebellar population.

Another region of the brain that is susceptible to the effects of ethanol in the early neonatal period is the hippocampus – particularly the CA1 region. Chronic exposure during the second trimester can produce permanent pyramidal cell deficits in the dorsal CA1 and the dentate gyrus of the hippocampus (Barnes and Walker, 1981, Klintsova et al., 2007). Chronic exposure to a high dose of ethanol during the early third trimester also produces CA1 cell loss (Bonthius and West, 1990, Tran and Kelly, 2003), and reduction in gross brain weights in the rat (Bonthius et al., 1988). These chronic – dose studies indicate the potential severity of ethanol, creating hippocampal neuronal deficits and gross microcephaly. Shorter duration binge studies, encompassing exposures up to 7 consecutive days in the third-trimester equivalent, have also been shown to result in permanent CA1 pyramidal cell deficits and it appears that like the Purkinje cell, the pyramidal cell also shows variable sensitivity to ethanol that is dependent on postnatal age (Bonthius and West, 1990, Livy et al., 2003, Tran and Kelly, 2003)
The Morris Water Maze (MWM) has been used to assess spatial learning and memory capacity of the hippocampus in numerous studies of both normal and brain damaged animals. Spatial learning deficits have been found in adolescent rats exposed to multiple ethanol binges on consecutive days during the second, but not the first neonatal week (Goodlett and Johnson, 1997). Further to this, a single ethanol binge produced a deficit in escape latency in rats tested during adolescence (Pauli et al., 1995b), an increase in total errors on a serial spatial discrimination reversal learning task (Thomas et al., 2004) and in reference memory acquisition during adulthood (Shoemack, 2007). These behavioural anomalies indicate that hippocampal function has been disrupted and thus it is likely that hippocampal structure has been altered by neonatal ethanol exposure. These behavioural changes most likely represent neuronal loss in the hippocampus, and a control-matched stereological study is the most reliable way to investigate the cellular corollary of such behavioural changes.

It must be noted however that although the hippocampus is an important centre for spatial learning and memory, and has important associations with parts of the prefrontal cortex and limbic system where acute cell death following a single ethanol binge has been detected, no studies to date have investigated the effect of a single ethanol binge in the early neonatal period on cell loss in the hippocampus.

3.1.1 Hypothesis

A single binge ethanol exposure on PN6 will result in a dose-dependent reduction in the number of CA1 pyramidal cells in the hippocampus.

3.1.2 Specific objectives

1. Establish a dose – response relationship of pyramidal cell loss after a single binge using the optical disector stereological technique.
2. Investigate the possible correlation between peak BEC and the number of pyramidal cells in the CA1 area.
3. Analyse the effect of a single ethanol binge on gross brain and body weights.
3.2 Results

3.2.1 Animals

Group numbers (n) are as follows; n (E6) = 8, n (E5) = 8, n (E4) = 8, n (IC) = 8.

All animals that were treated with alcohol, regardless of the ethanol dose, showed a decrease in responsiveness to normal handling a short time after the second ethanol delivery. Animals in the E6 and some in the E5 group showed signs of shaking, muscle spasms and decreased suckling on the dam. These animals were given extra top-up milk only intubations to compensate for a potential loss of nutrition. After 24 hours the majority of these animals appeared to be behaving normally.

3.2.2 Gross body weight

A one-way ANOVA (Figure 3.1) revealed no significant differences in bodyweight between treatment groups on PN6 prior to treatment (F=0.5415, P=0.658).

![Gross PN6 Body weights](image)

**Figure 3.1** Gross PN6 Body weights

There was no difference in mean body weight between treatment groups on PN6. Data represent mean ± SEM.
After treatment on PN6 animals in the E5 and E6 groups did not show weight gains to the same magnitude as the IC group for the next 4 days. In some cases pups lost weight over this period. This is commonly seen after a single binge with a high dose of ethanol, however these animals quickly recovered and by PN14 no difference in body weight between groups was evident.

Figure 3.2 Mean body weights during juvenile development

No differences were seen on PN6, however E6 and E5 groups showed significantly less body weight than the IC group on PN8 (P < 0.001, P < 0.05 respectively). By PN10 only the E6 group weighed less than the IC group (P < 0.05). From PN14 no differences in mean body weight between groups were evident. Data represent mean.
3.2.3 Blood Ethanol Concentration (BEC)

The mean peak BECs differed significantly between treatment groups by comparison of means using a one-way ANOVA ($F=13.76, p < 0.01$). The mean ± SD peak BEC for the E6 group was $486.1 ± 72.9$ mg/dl; E5 group $398.6 ± 53.5$ mg/dl; and E4 group $371.6 ± 30.34$ mg/dl. A Post-hoc test using the Newman-Keuls adjustment revealed a significant difference between the E6 and E4 groups ($p < 0.01$), and between the E6 and E5 groups ($p < 0.01$).

Figure 3.3 Peak BECs for ethanol treated animals

The E6 group showed significantly higher BEC levels than both the E5 and E4 groups. The E5 group was not different than the E4 group. Blood samples were taken 90 minutes after the second ethanol intubation as this has been shown to represent peak BEC. Data represent mean ± SEM. ** = Significant difference ($P<0.001$).
3.2.4 Gross Brain Weights

All whole brain, forebrain, cerebellar and brainstem weights were recorded immediately following perfusion on PN365 (Figure 3.4). A significant difference was found between the mean cerebellar weight (grams) of the E6 group compared with the IC group (E6 mean ± SD of 0.254 ± 0.050, compared with IC mean of 0.335 ± 0.045; P = 0.0092). Although not statistically significant, the whole brain weight and forebrain weight of the E6 animals showed a distinct reduction in weight compared to all other groups.
Figure 3.4 Gross brain weights

Mean weight (grams) of brain regions after ethanol (E) or control (IC) treatment. Data represent mean ± SEM. * E6 significantly different to IC (P < 0.01).
3.2.5 Hippocampal Pyramidal Neurons

There was a dose-dependent reduction in the number of pyramidal cells in the CA1 region of the hippocampus, as shown in Figure 3.5. A one-way ANOVA indicated a significant effect of treatment on the mean number of pyramidal cells \(F(3,28) = 22.21, \ p<0.0001\). Post-hoc analysis using the Newman-Keuls adjustment showed all alcohol groups had a significant reduction in CA1 pyramidal cells compared with the control group (Table 3.1). The E6 group also had significantly less cells than the E4 group (Table 3.1).

Table 3.1 Post-hoc analysis of the number of pyramidal cells in the CA1 layer of rat hippocampus

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Difference</th>
<th>q</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6vIC</td>
<td>-57782</td>
<td>10.98</td>
<td>P &lt; 0.001***</td>
</tr>
<tr>
<td>E5vIC</td>
<td>-44866</td>
<td>8.524</td>
<td>P &lt; 0.001***</td>
</tr>
<tr>
<td>E4vIC</td>
<td>-31366</td>
<td>5.959</td>
<td>P &lt; 0.001***</td>
</tr>
<tr>
<td>E6vE5</td>
<td>-12916</td>
<td>2.454</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>E6vE4</td>
<td>-26417</td>
<td>5.019</td>
<td>P &lt; 0.01**</td>
</tr>
<tr>
<td>E5vE4</td>
<td>-13500</td>
<td>2.565</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

All treatment groups were significantly different than the IC group (*** P<0.001), and the E6 group also showed a significant reduction in cells relative to the E4 group (** P<0.01).
**Figure 3.5** Total number of pyramidal cells in CA1

The total number of pyramidal cells in the CA1 region of the rat hippocampus at PD365 in each of the treatment groups. Solid lines indicate mean ± SEM. A one-way ANOVA revealed a significant effect of treatment (*** P < 0.0001, ** P < 0.001, Newman-Keuls test).

Absolute number of pyramidal cells in CA1 for each group are shown below in Figure 3.2. Note the mean number of cells in the E6 group is approximately half that of IC.

**Table 3.2** Mean, standard deviation and standard error of the mean distributions throughout the treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6</td>
<td>60800</td>
<td>15382</td>
<td>5438</td>
</tr>
<tr>
<td>E5</td>
<td>73716</td>
<td>15839</td>
<td>5600</td>
</tr>
<tr>
<td>E4</td>
<td>87217</td>
<td>12314</td>
<td>4354</td>
</tr>
<tr>
<td>IC</td>
<td>118582</td>
<td>15731</td>
<td>5562</td>
</tr>
</tbody>
</table>
A two-way ANOVA with sex and treatment as factors (Figure 3.6) did not reveal any effect of sex on CA1 neuronal number. Group numbers were E6 M = 3, F = 5; E5 M = 5, F = 3; E4 M = 5, F = 3; IC M = 3, F = 5.

**Figure 3.6** Total number of pyramidal cells in CA1 area in male and female animals

A 2-way ANOVA with treatment and sex as factors revealed a nearly significant interaction of P = 0.0686. This suggests that females in the E6 group may have been affected more by the alcohol than the males, resulting in reduced numbers of pyramidal cells however this would need to be tested using a larger number of animals in each experimental group.
A correlation analysis comparing the relationship between the number of CA1 pyramidal cells and the BEC of that animal was close to significance (Figure 3.7). This indicated a trend that animals with higher BEC values were likely to have less CA1 pyramidal cells.

**Figure 3.7 BEC / Pyramidal cell count correlation**

A two-tailed correlation P-value of 0.094 and $R^2$ value of 0.1223 indicate a trend of fewer CA1 pyramidal cells in animals treated with high dose alcohol, and thus a high BEC value. Each data point represents one animal. Control animals were not included in this figure as were not treated with alcohol (BEC = 0).
3.3 Discussion

The current study investigated the effects of a single ethanol binge on PN6 on the number of CA1 pyramidal cells in the hippocampus of the Long Evans rat. Tissue was collected when the animals were in adulthood, as the long-term consequences of binge like ethanol exposure on a single day in early neonatal life has not been documented.

Mean peak BEC values showed the E6 group differed significantly from the E5 and E4 group. This is important as any differences seen between treatment groups can be directly attributed to the differential ethanol doses, achieving different peak BEC levels. It is of interest to note that although the dose increased by an equivalent amount of g/kg ethanol, a consistent proportional increase in BEC was not evident – suggesting it may be important to determine the total blood ethanol profile for each dose. Both peak BEC and the duration of an elevated BEC beyond a critical level are important in acute ethanol toxicity in the developing nervous system (Li, 2005). Various methodologies are used to measure peak BEC values – including enzymatic assays and gas chromatography (used in all experiments in this thesis). The E6 group in this study registered a mean peak BEC of 486 mg/dl, while after the same dose Tomlinson and colleagues registered a mean peak BEC of only 390 mg/dl after using enzymatic assays (Tomlinson et al., 1998). It is generally considered that analysis using an ANALOX machine and by enzymatic assay kits underestimate the BEC levels relative to gas chromatography (Klintsova personal communication, R Napper). Therefore an underestimation of BEC levels could confound comparisons between studies, and perhaps this issue needs to be addressed in the wider alcohol research community, to enable reliable comparisons and meta-analysis to be undertaken in this very important field.

Gross brain weights between treatment groups did not differ, however when dissected into major brain regions the high dose ethanol group (E6) clearly showed reductions in weight, particularly in the cerebellum. All alcohol groups did show a trend of decreased weight in most brain regions, and additional animal numbers in each group would likely convert this to become significant. The brains of both male and female rats (balanced across treatment groups) were included in this sample, and due to a smaller body size of mature female rats and thus smaller brain, the increased variance within each group made detection of significant differences more difficult. No studies to date have shown
a single ethanol binge to cause damage of a magnitude that results in a gross brain
weight deficit. The E6 group showed a significant reduction in gross cerebellar weight
compared to the IC animals – a unique and important finding. This is also supported by
human neuroimaging data which show that FASD patients have reduced cerebellar
volume, along with the amygdala, basal ganglia and corpus callosum (Autti-Ramo,
2002). It is also likely that dissection of the forebrain into smaller sub regions would
show deficits in the E6 group as is likely to occur if the hippocampus had been
dissected and weighed. Previously, only high-dose binge administration to pups over a
number of consecutive days during the early post-natal period has produced reductions
in gross forebrain, cerebellar and brainstem weight (Bonthius et al., 1988). The
aforementioned study perfused animals on PN10 when the brain is still developing,
while animals in this experiment were mature adults – therefore recovery of gross
cerebellar microcephaly does not appear likely after a high dose binge. For a single
binge during the third trimester equivalent to produce such gross abnormalities,
widespread apoptotic neurodegeneration must be present. Goodlett and colleagues
administered binge like alcohol to rats on PN4 through 9 at a rate of 4.5g/kg/day, and
found reductions in both whole brain and cerebellar weights (Goodlett et al., 1991).
However it is important to note that this study shows that the lack of a regional brain
weight deficit does not mean there is not deficit of cells.

The current study has shown that a single binge ethanol exposure during development,
even when the ethanol dose is moderately low, can produce significant permanent cell
loss in the CA1 area of the hippocampus. All three alcohol-exposed groups used in this
study exhibited profound pyramidal cell loss in this important brain region, with
established roles in learning, memory and emotional response. It is important to
consider that the current study design did not employ the use of suckle control (SC)
animals to compare with the IC animals. The reasoning behind this was to date there
has been no literature to suggest that the intubation procedure itself causes neuronal loss
within any areas of the brain (Lee et al., 2008). As behavioural changes within control
groups were not always consistent (see Chapters 5-7), it may be prudent for future
studies of this nature to include SC groups in post-mortem studies for comparative
purposes so more robust conclusions can be made.

Previous studies have shown that CA1 pyramidal cells and place cells are severely
depleted after chronic ethanol exposure during prenatal – through postnatal
development (González-Burgos et al., 2006). Further to this, chronic ethanol exposure over a number of consecutive days during the third trimester equivalent results in a CA1-specific deficit in neuronal number, with relative sparing of the CA3 and dentate gyrus regions (Bonthius and West, 1990, Livy et al., 2003, Tran and Kelly, 2003). Human neuroimaging studies have shown reduced hippocampal volume in children diagnosed with FASD (Willoughby et al., 2008, Coles et al., 2011).

The current study indicates that gross damage can occur after only one binge, and this must be taken into consideration in future studies as the inclination is to over-expose the animals either through dose concentration or period of exposure. It is clear that long periods of exposure and very high doses can cause microcephaly and region-specific weight reductions, and the focus now must be on the threshold dose and duration for this damage to occur – as the present study has begun to elucidate. It is important to note that binge drinking on a single day followed by alcohol free days is a pattern of drinking seen in pregnant women, rather than daily drinking (Watson and McDonald, 1999b).

The mean number of CA1 pyramidal cells in the IC animals in the current study was less than numbers previously reported in the literature. The current study found a single hemisphere mean of 119000 in control animals (global mean of 238000), while West and colleagues (1991) found a global mean of 380000 CA1 neurons in 30 day old Wistar rats using the optical fractionator method (West et al., 1991). Boss and colleagues (1987) found a global mean of 320000 and 420000 CA1 neurons in 30 day old Wistar and 30 day old Sprague-Dawley rats respectively using the Abercrombie correction, while Seress (1988) found 240000 and 260000 CA1 neurons in 3-4 month old Sprague-Dawley and CFY rats also using the Abercrombie correction (Boss et al., 1987, Seress, 1988). The estimate calculated in the current study was slightly lower than these estimates – one explanation for this is the age of the animals used – 365 days, which is substantially older than all of the aforementioned studies. Also the methods of delineation used here were very stringent with regard to the neurons counted (pyramidal neurons, not glia cells or other neurons) and the area of delineation. Many previous estimates include large portions of the subiculum due to the difficulty in delineating it from the CA1 layer, however the current study marked the beginning of the CA1 as where the subiculum has fully tapered off to the compact CA1 layer. Both of these factors are likely to have contributed to the relatively low estimate, and importance
should be placed on the relative neuronal deficits seen in the ethanol treated animals rather than on comparisons between control animals and previous data.

As a significant proportion of cells have been deleted from the CA1 area following this PN6 ethanol binge, both efferent and afferent connections, and the structures which these synapse with may be induced to delete cells via apoptosis to compensate for the lack of inputs or neurons to synapse with their outputs and maintain numerical ratios (Herrup and Sunter, 1987, Herrup et al., 1996). The hippocampus is not the only area of the brain vulnerable to the effects of ethanol. Although acute cell death does occur in the hippocampus it is possible that the reduction in neuronal number seen here is due in part to neuronal matching of CA1 neurons to neurons in other regions that have also been affected, with this occurring at some time after the acute ethanol-induced apoptotic cell death.

3.4 Conclusion

The third trimester appears to be a very specific temporal window of vulnerability for particularly the hippocampus, and it is clear only a single ethanol binge at a moderate dose can have long lasting effects on hippocampal structure at this time. It is reasonable to assume that if such a large loss of cells in this learning and memory centre of the brain occurs, a subsequent loss of function in tasks related to hippocampal or interconnected structures may be observed. As the hippocampus is intricately connected with other regions of the brain including the prefrontal cortex – an area critical for social functioning – it is of interest to investigate cell loss in this area also.
Chapter 4. Acc Stereological Investigation

4.1 Introduction

A single binge dose of ethanol administered on PN6 produces profound pyramidal cell deficits in the CA1 area of the hippocampus in a dose–dependent fashion (see chapter 3). Even the lowest dose of 4.5g/kg produced a significant reduction, and indicates that high doses are not necessary to cause permanent damage. This has many implications, including putative loss of input/output to and from other centres of the brain, or rewiring of existing circuits to compensate for an altered flow of information. Intrinsic and extrinsic connections may also suffer similar patterns of cell loss to normalize hippocampal circuitry and function.

While a loss of spatial learning and memory in children with FASD may create huge difficulties, it is the ability to carry out executive functions, emotional and social understanding which can make or break a child in society and adulthood. The profound hippocampal neuronal loss demonstrated in the previous chapter does not result in functional deficits as severely as expected (Shoemack, 2007). This suggests that the intrinsic and extrinsic connections of the hippocampus show cell loss with binge ethanol exposure to maintain cell circuit integrity.

The Acc has been shown to be acutely vulnerable to a single binge dose of ethanol in both the rat and mouse (Figure 4.1). Olney and colleagues found that massive acute cell death (45%) was present in the cingulate cortex of the mouse on PN10, 72 hours after ethanol treatment (Olney et al., 2002b).
Figure 4.1 Pattern of ethanol-induced caspase-3 activation in the rostral forebrain following subcutaneous injection with saline

The cingulate cortex (Cing) in the ethanol-exposed animal (right) shows many more neurons with activated caspase-3, indicative of programmed cell death. The few neurons in the saline-treated animal (left) showing caspase-3 activation can be attributed to normal levels of cell death in the developing mouse brain. Adapted from Olney et al. (2002).

Mice exposed to only one binge ethanol dose on PN7 also show a smaller volume of the retrosplenial cortex (otherwise known as the posterior cingulate cortex), the anterior thalamic nuclei and the mammillary bodies – all of which are components in the extended hippocampal circuit (Wozniak et al., 2004).

Acute cell death represented as a significant increase in apoptotic cell density after a single ethanol binge on either PN4 or PN6 has also been documented in the rat Acc (Genge, 2006). However assessment of Acc cell death in the mature brain after a single postnatal ethanol binge has not been undertaken. This is of particular importance, as the cingulate cortex has numerous intrinsic connections with the CA1 area of the hippocampus (Jay et al., 1989, Jay and Witter, 1991).
If permanent cell loss occurs in the Acc this would add to our understanding of which neuronal networks are affected directly or indirectly as a result of exposure to alcohol during development.

4.1.1 Hypothesis

A single ethanol binge on PN6 will result in a dose–dependent reduction in the total number of neurons in the anterior cingulate cortex. This loss will parallel that of the hippocampal CA1 cell loss previously found in a dose dependent fashion.

4.1.2 Specific objectives

1. Establish a dose–response relationship of long-term neuronal loss after a single ethanol binge on PN6, using the optical disector stereological technique.
2. Investigate the possible correlation between peak BEC and the number neurons in the Acc.

4.2 Results

4.2.1 Animals and Blood Ethanol Concentration (BEC)

Group numbers (n) were as follows; n (E6) = 7, n (E5) = 8, n (E4) = 8, n (IC) = 8. The tissue sections used for the Acc investigation were from the same animals as used in the CA1 stereology and therefore the BECs values remain the same. The mean peak BECs were shown to differ significantly between treatment groups by comparison of means using a one-way ANOVA (F=13.76, p<0.01). The mean peak BECs differed significantly between treatment groups by comparison of means using a one-way ANOVA (F=13.76, p<0.01). The mean ± SD peak BEC for the E6 group was 486.1 ± 72.9 mg/dl; E5 group 398.6 ± 53.5 mg/dl; and E4 group 371.6 ± 30.34 mg/dl. A Post-hoc test using the Newman-Keuls adjustment revealed a significant difference between the E6 and E4 groups (p < 0.01), and between the E6 and E5 groups (p < 0.01).
4.2.2 Neuronal cell number in Acc

The entire rostral part of the cingulate cortex (the anterior cingulate cortex, Acc), was sampled in a random systematic fashion and the number of neurons was determined using the optical fractionator stereological method. There was a significant effect of treatment on total neuron number within the Acc (one-way ANOVA, \( F = 6.167, P = 0.0025 \)). Post-hoc analysis using the Newman-Keuls test showed a significant reduction in total cell number in the E6 and E5 groups relative to IC, E6 relative to E4, and E5 relative to E4 as shown in Table 4.1 below.

Table 4.1 Newman-Keuls post-hoc analysis of the total number of cells in the Acc

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Difference</th>
<th>q</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6vIC</td>
<td>-133049</td>
<td>5.002</td>
<td>P &lt; 0.01**</td>
</tr>
<tr>
<td>E5vIC</td>
<td>-110519</td>
<td>4.3</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>E4vIC</td>
<td>-21716</td>
<td>0.845</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>E6vE5</td>
<td>-22530</td>
<td>0.8469</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>E6vE4</td>
<td>-111333</td>
<td>4.185</td>
<td>p &lt; 0.05*</td>
</tr>
<tr>
<td>E5vE4</td>
<td>-88803</td>
<td>3.455</td>
<td>p &lt; 0.05*</td>
</tr>
</tbody>
</table>

Both E6 and E5 groups showed significantly less cells in the Acc than the IC group (*P<0.05 and **P<0.01 respectively). The E6 and E5 groups also had less cells than the E4 group (*P<0.05) (Figure 4.2).
**Figure 4.2** Number of neurons in Acc by treatment group

Solid coloured lines indicate mean ± SEM. * = significant difference between E6 and E4, E5 and IC and E5 and E4 groups with P<0.05, ** = significant difference between E6 and IC groups with P<0.01.
Absolute values for the number of neurons in the Acc for each group are displayed in Table 4.2 below. The E5 group had a slightly higher standard deviation that the other three groups, however still had significantly less neurons than both IC and E4 groups.

Table 4.2 Mean, standard deviation and standard error of the mean distributions throughout the treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6</td>
<td>335795</td>
<td>54538</td>
<td>5438</td>
</tr>
<tr>
<td>E5</td>
<td>358325</td>
<td>93225</td>
<td>32960</td>
</tr>
<tr>
<td>E4</td>
<td>447128</td>
<td>72130</td>
<td>62749</td>
</tr>
<tr>
<td>IC</td>
<td>468844</td>
<td>62749</td>
<td>22185</td>
</tr>
</tbody>
</table>

Unlike the CA1 cell count study in chapter 3, the E4 group did not show a significant difference in numbers of Acc neurons over the IC group.
A two-tailed correlation analysis comparing the relationship between the number of neurons in the Acc and the peak BEC revealed a significant correlation ($R^2 = 0.18$, $P < 0.05$). Therefore as peak BEC increases, the cell number in the Acc decreases (Figure 4.3).

**Figure 4.3** Correlation analysis between peak BEC and number of neurons

This figure shows an inverse relationship between the peak BEC and the total number of neurons in the Acc. This is important for determining cell loss after various degrees of alcohol exposure that may have not been investigated in animal models.
4.3 Discussion

The present study hypothesized that following a single ethanol binge on PN6, the Acc would show a dose–dependent reduction in the number of neurons present. This was expected, as in chapter three it was found that a dose-dependent depletion in CA1 cells of the hippocampus in mature animals was evident after this binge exposure. A number of lines of evidence suggest the Acc would demonstrate significant cell loss. Apoptotic cell death occurs in this region following an acute ethanol exposure in early postnatal life (Olney, 2004b) and as these two areas are highly interconnected, it was reasonable to assume the Acc may have sustained a cell loss to ensure that quantitative relationships are maintained within the neuronal circuits of the forebrain and hippocampus.

This hypothesis was supported with a significant dose–dependent reduction of the neurons that make up the Acc. Both E6 and E5 groups exhibited a massive neuronal deficit relative to control animals, indicating that a single dose of ethanol can result in permanent damage to the Acc, an important executive centre of the brain. The E4 group did not show a significant reduction in neurons within the Acc relative to the IC group, but they did show a significant reduction in the hippocampal CA1 neurons. It is possible that the hippocampus is more sensitive to the neurotoxic effects of ethanol than the Acc. It is also feasible that the “neuronal matching” mechanism has a threshold level of cells required to be deleted before connected regions delete neurons to match the altered input or output. It may also indicate that in the E4 animals where a smaller percentage of Acc neurons were lost there has been other compensatory changes occurring to ensure the circuit stoichiometry is optimized. Different sensitivities to the neurotoxic effects of an acute ethanol dose has been demonstrated by differences in the magnitude of Purkinje cell death between cerebellar vermal lobules, with the same dose on the same day (Bonthius and West, 1990).

Chronic prenatal alcohol exposure throughout gestation has shown a 45% reduction in GABAergic neuronal number in the Acc – a similar reduction seen in this study, although the type of cells counted, ethanol exposure and delivery differed (Moore et al., 1998). However, a study by Mitchell and colleagues (2000) did not find any differences in the number of GABAergic neurons in the Acc between ethanol exposed and control animals (Mitchell et al., 2000). Alcohol was administered during PN4 through 10 of the
brain growth spurt, and cells counted during adulthood – similar to the current study, however again only GABAergic parvalbumin-immunoreactive neurons were localized and counted, which does not represent the entire neuronal population of the Acc, which is composed of many types of neurons. This study has shown that a correlation exists between the peak BEC level and the number of neurons present in the Acc. This confirms that the ethanol neurotoxicity causing apoptotic cell death is acting in a linear, dose-dependent fashion. This is crucial to the way data such as these must be portrayed to at-risk mothers, as it is clear that as the concentration of alcohol consumed during only a single session increases, the number of neurons dying in the Acc of the foetus is also likely to increase.

Literature on neuronal numbers in the mature Acc following neonatal alcohol exposure is sparse. The findings from this study indicate that the Acc is vulnerable in a similar way to the hippocampus after exposure during only as single day in the brain growth spurt. This is a very important finding as no previous work has shown permanent neuronal loss in this part of the Acc in the brain on such a scale, and in a dose-dependent fashion. With such large numbers of mature neurons lost from the Acc, the functions of this area – such as social behaviour, inhibitory control and other complex cognitive executive functions are possibly altered.

It is important to note that the significant deficit in Acc cell loss occurred when the mean peak blood ethanol concentration was around 400mg/dl or higher. This can be equated to around 250mg/dl, based on comparative LD50 ethanol doses in the rat and human, as rats have a higher metabolic rate and hence BECs rise quicker after comparable doses in humans. These ranges of BECs can occur in females after only 4-5 standard drinks if consumed rapidly, as each standard drink containing 10g of ethanol has a cumulative effect if the liver does not have adequate time to metabolise it (approximately one standard drink per hour).

4.4 Conclusion

A single ethanol binge on PN6 produced significant cell loss in the Acc in E5 and E6 groups relative to IC animals. This is important, as along with the hippocampal CA1 cell loss, it appears these two interconnected areas are vulnerable to cell loss and
therefore may be susceptible to functional deficits also. The Acc has been implicated in adequate social play functioning in rats, and this may be compromised if circuit integrity and a reduction in neuron number have occurred.
Chapter 5. PN32-34 Play Behaviour Analysis

5.1 Introduction

Chapters 3 and 4 have shown that a single binge on PN6 can produce significant cell loss in both the CA1 area of the hippocampus, and in the Acc – in a dose dependent manner. Both of these different brain regions are highly interconnected, therefore a loss of cells in both regions may cause alterations in both the functional output and synaptic relationships with the thalamus, limbic system and somatosensory system to name a few.

The Acc is an integral initiator and modulator of various executive, cognitive and social functions. If the Acc has lost up to 40% of the pyramidal neurons crucial to this output, then it is feasible that some of its functional capacity has been lost also. Children with FASD have been shown to behave abnormally during various social situations, and it is these social contexts which form the foundations for social behaviour and understanding later in life (Coles et al., 1997, Autti-Rämö, 2000, Korkman et al., 2003, Salmon, 2008, Greenbaum et al., 2009). Play fighting during adolescence is one area of social behaviour that is crucial for normal development and adult behaviour of the rat, and is directly linked to the Acc and its associated prefrontal-hippocampal-thalamic networks. Lesions to the medial prefrontal cortex (mPFC) produce a specific change in play fighting defensive strategies from more complex (supine defence) to less complex (evasive defence) manoeuvres (Bell et al., 2009). The experience of play during adolescence is crucial for social development and functioning later during maturity in the rat. Social isolation during adolescence results in a reduction in play during adulthood, suggesting that proper social functioning and exposure during adolescence is critical for the development of normal play activity once maturity is reached (Hol et al., 1999).

The mPFC is also one of the cortical areas involved in initiation and motivational factors related to rough and tumble play, hence damage to this region may not only produce differences in defensive strategies, but whether the animal shows normal frequencies of attack and defence (Vanderschuren et al., 1997, Bekkedal et al., 1998). Play fighting is disrupted in rats exposed to neonatal ethanol in the form of deficits in somatosensory processing manifesting as reduced sensitivity to nape attacks. Also,
these alcohol exposed animals show an increase in the frequency of supine defense when attacked (Lawrence et al., 2008), which differs from mPFC lesioned rats which show an increase in evasive defense strategies. This is interesting as both of these studies use animals that have some damage to the mPFC, however show disparate results in relation to defensive responses. As documented in the previous chapter, if Acc damage has occurred in alcohol exposed animals in the form of cell loss, then some form of anomaly in play behavior is expected, whether it be changes in the frequency of attack and defense, or in the type of defense elicited in response to an attack.

5.1.1 Hypothesis
A single ethanol binge on PN6 will result in abnormalities in adolescent play behaviour, manifest as hyperactivity and atypical defensive tactics, in a dose-dependent manner.

5.1.2 Specific objectives
1. To use a well documented play behaviour and analysis paradigm to investigate the rough and tumble aspect of social behaviour after a single ethanol binge on PN6 in a controlled setting.
2. To determine if there is a dose-response effect of a single binge ethanol exposure on PN6 on adolescent play behaviour, with E6 and E5 animals more affected due to a greater loss of Acc neurons.

5.2 Results

5.2.1 Animals
Group numbers (n) were; n (E6) = 11, n (E5) = 12, n (E4) = 9, n (IC) = 10, n (SC) = 47. After intubation, animals in the alcohol-exposure groups showed signs of inebriation and unresponsiveness for around 4 hours post peak ethanol, similar to animals in the cell count studies. Occasionally an E5 or E6 animal died either as a result of the intubation or of the subsequent intoxication. When this occurred the animal was immediately removed from the home cage. Treatment group numbers were balanced across the 3 cohorts of animals, used to accommodate any pup losses.
5.2.2 Gross body weight

A one-way ANOVA revealed no significant differences in body weight between treatment groups on PN6 (F=0.387, P=0.817).

![Mean PN6 body weights](image)

**Figure 5.1** Mean PN6 body weights

No differences were found between groups following a one-way ANOVA (F=0.387, P=0.817). Data represent mean ± SEM.

Similar to the stereology cohort of animals, the E6 and E5 groups showed significantly lower weights on PN8 and PN10 than SC and IC animals. PN8 mean body weight for the E6 group were significantly different to both SC (P < 0.001) and IC animals (P < 0.05). E5 animals were also significantly less than the SC group (P < 0.05) on PN8. E6 animals also weighed significantly less than SC (P < 0.001) and IC (P < 0.01) animals on PN10. E5 animals showed a similar difference to both SC (P < 0.01) and IC (P < 0.05) animals on PN10.

From PN14 onwards these weight deficits had been eliminated and all groups showed similar growth levels until weaning on PN25 (Figure 5.3).
Figure 5.2 Mean body weights during juvenile development

No weight differences were present on PN6, however both E6 and E5 groups showed a significant weight deficit when compared to SC and IC groups on PN8 and PN10. Data represent mean.

5.2.3 Blood ethanol concentrations

As for the animals used in the stereological investigations, blood samples were taken at 90 minutes after the second ethanol intubation. No statistically significant differences in BECs were found between the E6, E5 and E4 groups in this cohort. This was interesting to note, as the mean values for E6 and E5 groups were noticeably higher than the E4 group, however a high level of variability resulted in a non-significant one-way ANOVA for mean peak BEC. Some extreme values in each of the ethanol treated groups prevented a significant difference relative to E4.
These values as seen in Figure 5.2, were not more than 2 standard deviations from the mean and so could not be considered as outliers and therefore were included in the analysis. Although here was no difference in variance between groups (Bartlett’s test, $P = 0.5447$), the unusual variability in the peak BEC values found after a dose of 4.5g/kg will have contributed to this result. All due care was taken to ensure accurate results in both the timing and collection of blood for BEC analysis, therefore the anomalies must have arisen from differences in internal metabolic rates, absorption, congenital anatomical or physiological processes between these animals.

Figure 5.3 Blood ethanol concentration by treatment group

A one-way ANOVA revealed no significant difference in peak BEC between groups ($P = 0.1387$). A Bartlett’s test for equal variances produced a non-significant result ($P = 0.5447$), indicating the variability of each treatment group was the same, thus validating the use of the one-way ANOVA.
5.2.2 Qualitative Observations

All play fighting testing and analysis was carried out with the experimenter blind to the treatment group. However after analysis was finished some codes were broken to allow subjective observations. It was noted that animals which had particularly high BEC values were very hyperactive, and along with an increase in the rate of attack, consistently reared up against the side of the chamber and jumping in an attempt to escape.

5.2.4 Social play fighting analysis

Play fighting data was analysed using one-way ANOVA, two-way ANOVA and a linear mixed model (LMM). The ANOVA models were used to analyse differences in group means and variability, while the LMM was used to assess 2 levels of correlation – both within animal and between animal. The LMM gives the freedom to model not only the mean of a response variable, but also the covariance structure.

Analysis of play behaviour is complex as in each play period analysed, each animal will behave as an attacker and as a defender. Within any play period where at least one animal is a SC animal, whether an animal acts as an attacker in the first interaction with the SC animals or a defender may influence the overall behaviour of that pairing. Also whether each animal in a group has a tendency to show a preference for being an attacker or a defender will influence the overall behaviour of the group. For this reason all data was analysed using two statistical methods; one-way ANOVA and a linear mixed model (LMM) on Stata using the XT mixed command. The LMM allows for the fact that there are at least two levels of variation – each animal is both an attacker and defender - and therefore subtle individual differences relative to the group, rather than just group alone, are taken into account. The LMM model has a random effect for the attacker and a random effect for the defender, which allows for different levels in aggressiveness and other individual traits. This produces a more accurate biological model that represents the data in a way that simple non-repeated measures ANOVAs cannot, as these only take the group variance into account.
5.2.3.1 Attack and defence

The mean number of attacks in total for each pair during each ten-minute play session was determined and analysed using a one-way ANOVA (P < 0.05). As can clearly be seen below all treatment groups, including the IC group showed higher rates of attack than the SC group.

![Bar chart showing mean number of attacks for each treatment combination](image)

**Figure 5.4** Mean number of attacks for each treatment combination

Each bar represents the mean total number of attacks in each ten-minute session for each treatment and SC pair. Data represent mean ± SD. A Newman-Keuls post-hoc test revealed differences between E6+SC/SC+SC * (P<0.05), E5+SC/SC+SC * (P<0.05), E4+SC/SC+SC * (P<0.05) and IC+SC/SC+SC * (P<0.05).
A subsequent scatter plot (Figure 5.5) of the above data showed a number of extreme values in the three ethanol-exposed groups, relative to the tight cluster of the SC group.

**Figure 5.5** Scatter plot of total number of attacks for each pair

Each individual symbol represents the number of attacks made in the ten-minute period for each animal within the pair. Compare this with Figure 5.6 below, and it is evident that the animals which produced an extremely high attack count were the animals treated with some level of alcohol.
Figure 5.6 Mean attacks made by treatment groups on SC group

This figure shows the number of attacks made on SC animals by each treatment group. Interestingly, only the IC group was shown to attack more than SC animals *(P < 0.05). All other groups did however show a trend towards attacking more.

One-way ANOVA indicated that only the IC group made more attacks on the SC animals *(P < 0.05) (Figure 5.6). However LMM analysis of the number of attacks made by each group relative to SC animals per 10-minute play session revealed all ethanol-treated groups, and the IC group, all attacked more on average (Table 5.1). IC animals attacked 18.34 times more on average *(P < 0.01); E4 attacked 14.19 times more *(P < 0.05); E5 attacked 14.50 times more on average *(P < 0.01) and E6 animals attacked 13.58 times more on average *(P < 0.05). The analysis using LMM appears to reflect what is suggested in Figure 5.6 and may suggest that the LMM model is better suited to this type of data analysis, as the residual error (unexplained variation) is lower due to separate random effects for the attacker and defender. For this reason both ANOVA and LMM analysis are included, however the discussion of data will place emphasis on the LMM data analysis.
Table 5.1 LMM analysis of number of attacks per 10-minute period by treatment group, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>18.34</td>
<td>5.42</td>
<td><strong>0.001</strong></td>
<td>7.72 - 28.96</td>
</tr>
<tr>
<td>E4</td>
<td>14.19</td>
<td>6.00</td>
<td><strong>0.018</strong></td>
<td>2.44 - 25.95</td>
</tr>
<tr>
<td>E5</td>
<td>14.5</td>
<td>5.33</td>
<td><strong>0.007</strong></td>
<td>4.05 - 24.95</td>
</tr>
<tr>
<td>E6</td>
<td>13.59</td>
<td>5.53</td>
<td><strong>0.014</strong></td>
<td>2.75 - 24.42</td>
</tr>
</tbody>
</table>

Figure 5.7 Mean attacks made by SC on each treatment group

SC (E6) represents the mean number of attacks made by SC animals on E6 animals per 10-minute play session. It appears that SC animals attack all treatment groups, particularly E4 and IC animals more (P < 0.05). However Newman Keuls post-hoc analysis did not reveal any significant differences between individual groups.
As seen in Figure 5.7, a one-way ANOVA was used to determine if SC animals show a different rate of attack on animals from each treatment group, rather than a suckle animal, over the 10-minute play session. A significant effect of treatment was found (P < 0.05), however a post-hoc test using the Newman-Keuls adjustment did not show any significant individual differences. However the graph suggests that there may be a tendency for the SC animals to attack the E4 and IC animals a little more than other groups.

LMM analysis (Table 5.2) also found no differences between groups – although it does appear that the SC animals showed a tendency to attack E4 animals more (P = 0.078), with a coefficient of 8.17 more attacks.

**Table 5.2** LMM analysis of number of attacks on each treatment group by SC animals, relative to other SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>4.66</td>
<td>4.24</td>
<td>0.272</td>
<td>(-3.65 - 12.97)</td>
</tr>
<tr>
<td>E4</td>
<td>8.17</td>
<td>4.64</td>
<td>0.078</td>
<td>(-0.91 - 17.26)</td>
</tr>
<tr>
<td>E5</td>
<td>4.34</td>
<td>4.02</td>
<td>0.280</td>
<td>(-3.54 - 12.21)</td>
</tr>
<tr>
<td>E6</td>
<td>4.84</td>
<td>4.17</td>
<td>0.246</td>
<td>(-3.34 - 13.01)</td>
</tr>
</tbody>
</table>
The probability of defence in response to an attack (either an evade, partial or supine response) (Figure 5.8) was analysed using a one-way ANOVA and showed no differences in the average probability of defence, measured as a percentage ($P = 0.278$).

![Figure 5.8 Probability of defence during each 10 minute play session](image)

All groups showed very high rates of defence in response to an attack, and no significant differences were found following a one-way ANOVA ($P = 0.278$).

The way in which an animal responds to attack, namely how it defends, can be very informative. Thus each defence event was analysed and placed into one of three distinct categories; evasion where the defender was able to move the nape of the neck away from the attacker; partial defence where the defending animal rotated along the long axis of its body and while remaining with at least one hind-limb on the floor attempted to push the attacker away; supine defence where the defender rolled into a supine position with limbs no longer in contact with the floor.
The probability of supine defence when each treatment group was the defender revealed some interesting findings following a one-way ANOVA (P < 0.001). IC animals had a higher percentage of supine than SC (P < 0.001), E5 (P < 0.01) and E6 (P < 0.05). E4 animals also went supine more than SC (P < 0.01) and E5 (P < 0.05).

**Figure 5.9A** Percentage supine response when a defender

The probability in percentage that each group went into a supine position when defending against an attack. One-way ANOVA showed a significant effect of treatment (P < 0.001).

LMM analysis (Table 5.3) showed similar results to the one-way ANOVA, with both IC (P < 0.05) and E4 (P < 0.05) groups going supine on average 5.80 and 6.39 times more per 10-minute play session that SC animals.
Table 5.3 LMM analysis of supine defence per 10-minute period by treatment group, when compared with SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>5.8</td>
<td>2.45</td>
<td><strong>0.018</strong></td>
<td>(0.99 - 10.60)</td>
</tr>
<tr>
<td>E4</td>
<td>6.39</td>
<td>2.68</td>
<td><strong>0.017</strong></td>
<td>(1.14 - 11.63)</td>
</tr>
<tr>
<td>E5</td>
<td>2.18</td>
<td>2.34</td>
<td><strong>0.351</strong></td>
<td>(-2.40 - 6.76)</td>
</tr>
<tr>
<td>E6</td>
<td>2.94</td>
<td>2.43</td>
<td><strong>0.225</strong></td>
<td>(-1.81 - 7.70)</td>
</tr>
</tbody>
</table>

One-way ANOVA revealed no significant effect of treatment on the amount of supine defences elicited in their play partner when each treatment group attacked an SC animal (P = 0.090) (Figure 5.9B).

Figure 5.9B Percentage supine response elicited in the SC defender when an attacker

The probability in percentage, of a supine defence being elicited in the defending group (SC) by the attacking animal. One-way ANOVA revealed no significant effect of treatment (P = 0.090).
However LMM analysis (Table 5.4) indicated IC (P < 0.05), E4 (P < 0.05) and E5 (P < 0.05) groups all elicited more supine defences than SC animals.

**Table 5.4** LMM analysis of supine defence elicited in play partner, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>7.69</td>
<td>2.47</td>
<td>0.002</td>
<td>(2.84 - 12.53)</td>
</tr>
<tr>
<td>E4</td>
<td>5.21</td>
<td>2.63</td>
<td>0.048</td>
<td>(0.05 - 10.37)</td>
</tr>
<tr>
<td>E5</td>
<td>7.46</td>
<td>2.4</td>
<td>0.002</td>
<td>(2.76 - 12.16)</td>
</tr>
<tr>
<td>E6</td>
<td>3.25</td>
<td>2.49</td>
<td>0.192</td>
<td>(-1.63 - 8.12)</td>
</tr>
</tbody>
</table>

A one-way ANOVA revealed no significant effect of treatment on the probability of partial defence when each treatment group was the defender (P = 0.059) (Figure 5.10A). Further to this, no differences were found in probability of partial defence elicited between groups (P = 0.317) (Figure 5.10B).

![Figure 5.10](image-url) Percentage partial response when defender (A) and attacker (B)
Subsequent LMM analysis showed that when acting as the defender (Table 5.5), none of the treatment groups showed any significant difference in rate of partial defence over the SC group.

**Table 5.5** LMM analysis of partial defence by treatment group, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>-0.96</td>
<td>1.42</td>
<td>0.499</td>
<td>(-3.75 - 1.82)</td>
</tr>
<tr>
<td>E4</td>
<td>2.02</td>
<td>1.58</td>
<td>0.202</td>
<td>(-1.08 - 5.13)</td>
</tr>
<tr>
<td>E5</td>
<td>0.55</td>
<td>1.34</td>
<td>0.681</td>
<td>(-2.07 - 3.17)</td>
</tr>
<tr>
<td>E6</td>
<td>0.18</td>
<td>1.4</td>
<td>0.899</td>
<td>(-2.56 - 2.92)</td>
</tr>
</tbody>
</table>

Contrary to the one-way ANOVA, rates of partial defence elicited by treatment groups in SC animals (Table 5.6) were higher when the IC (P < 0.05) and E6 (P < 0.05) groups were the attacker than when SC animals were the attacker.

**Table 5.6** LMM analysis of partial defence elicited in play partner, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>5.18</td>
<td>2.62</td>
<td>0.048</td>
<td>(0.50 - 10.31)</td>
</tr>
<tr>
<td>E4</td>
<td>4.91</td>
<td>2.98</td>
<td>0.099</td>
<td>(-0.93 - 10.75)</td>
</tr>
<tr>
<td>E5</td>
<td>2.85</td>
<td>2.58</td>
<td>0.270</td>
<td>(-2.21 - 7.91)</td>
</tr>
<tr>
<td>E6</td>
<td>5.98</td>
<td>2.68</td>
<td>0.026</td>
<td>(0.73 - 11.23)</td>
</tr>
</tbody>
</table>
The probability of evasive defence by each treatment group in response to an attack by an SC animal was not different between any groups (P = 0.088) (Figure 5.11A). There was also no significant difference in the probability of the treatment groups eliciting an evasive defence in SC animals (P = 0.985) (Figure 5.11B).

**Figure 5.11** Percentage evasive response when defender (A) and attacker (B)

One-way ANOVA found no significant differences between groups for evasive defence when either defending (A) or attacking (B) (P = 0.088, 0.985 respectively).
LMM analysis agreed with this finding, with no groups evading significantly more than the SC group in response to an attack.

**Table 5.7** LMM analysis of evasive defence by treatment group, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>-0.53</td>
<td>1.39</td>
<td><strong>0.703</strong></td>
<td>(-3.24 - 2.19)</td>
</tr>
<tr>
<td>E4</td>
<td>-0.69</td>
<td>1.46</td>
<td><strong>0.636</strong></td>
<td>(-3.54 - 2.16)</td>
</tr>
<tr>
<td>E5</td>
<td>1.07</td>
<td>1.32</td>
<td><strong>0.421</strong></td>
<td>(-1.53 - 3.66)</td>
</tr>
<tr>
<td>E6</td>
<td>0.73</td>
<td>1.37</td>
<td><strong>0.594</strong></td>
<td>(-1.96 - 3.42)</td>
</tr>
</tbody>
</table>

However a LMM analysis (Table 5.8) indicated that evasion was elicited significantly more in the SC animals, when each of the treatment groups acted as the attacker. This indicates the SC animals were evading attacks from the treatment animals a lot more than treatment animals were evading attacks from SC animals (IC P < 0.001; E4 P < 0.01; E5 P < 0.01; E6 P < 0.05).

**Table 5.8** LMM analysis of evasive defence elicited in play partner, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>5.68</td>
<td>1.64</td>
<td><strong>0.001</strong></td>
<td>(2.46 - 8.89)</td>
</tr>
<tr>
<td>E4</td>
<td>4.77</td>
<td>1.83</td>
<td><strong>0.009</strong></td>
<td>(1.19 - 8.35)</td>
</tr>
<tr>
<td>E5</td>
<td>4.28</td>
<td>1.60</td>
<td><strong>0.007</strong></td>
<td>(1.14 - 7.41)</td>
</tr>
<tr>
<td>E6</td>
<td>4.24</td>
<td>1.66</td>
<td><strong>0.011</strong></td>
<td>(0.99 - 7.50)</td>
</tr>
</tbody>
</table>
5.3 Discussion

The present study used a form of social interaction innate to rats – play fighting – as a behavioural assay to investigate possible changes in social behaviour and acquisition of appropriate motor responses consequent of exposure to ethanol on PN6. Although play fighting is frequent, it is a sporadic activity that tapers off over time. Therefore the data presented was that occurring in the first 10 minutes of the pair of animals interacting after 24 hours of isolation. Another aspect of the study paradigm ensured all animals were familiar with their play partner prior to testing, as animals were housed in a quadrant with their play-fighting partner since weaning at PN25.

In contrast to the stereological studies, the mean peak BEC values in this behavioural cohort of animals did not reveal any statistically significant differences between treatment groups. This was due to a number of abnormal outliers in each treatment group, and subsequently increased the variance and affected the overall mean value. This was unexpected as all procedures, timing and volume of blood taken were identical to previous cohorts. It is possible these outliers had abnormally high or low metabolic rates, or altered liver enzyme levels which caused a down or up-regulation of ethanol metabolism. However, although no significant difference was shown between groups, the E6 group and E5 group still exhibited higher mean values than the E4 group – and this implies that the higher doses were still producing higher concentrations of ethanol in the bloodstream.

This study revealed that pairing of E6/SC, E5/SC, E4/SC and IC/SC, produced more attacks on average than the SC/SC pairing. Further to this, the LMM analysis showed that all treatment groups, including IC initiated more attacks on their SC counterparts than the SC initiated on them. It is possible that an animal within a pair that is more likely to attack may be more physically active than its pair-mate. This is consistent with studies that have shown hyperactivity in neonatally alcohol exposed rats (Royalty, 1990, Thomas et al., 1998b, Lugo et al., 2003) and children (Coles et al., 1997). The study by Royalty and colleagues (1990) used a 2/g/kg treatment regime throughout the gestation period, and showed the ethanol-exposed offspring not only elicited more playful responses, but increased aggression and hyperactivity during maturity also. The play behaviour findings are similar to this study – and suggest that a single post-natal binge may produce the same effects on social play as a chronic binge over multiple
days. The current findings of an increase in attack frequency are in contrast to that of Mooney and Varlinskaya (2011), who investigated the effects of a single ethanol binge on GD12 on social parameters. They found that only male animals exposed to ethanol displayed a reduction in play fighting behaviour, contact behaviour and social investigation during both adolescence and adulthood (Mooney and Varlinskaya, 2011). The same authors also conducted a study in 2014 on the effect of ethanol exposure on gestational day 15 on social motivation. A reduction in social investigation during adolescence was evident, however did not find any effect of ethanol on play fighting behaviour during adolescence or adulthood (Varlinskaya and Mooney, 2014).

Comparisons with this study are difficult due to the timing of alcohol exposure (gestational versus post-natal), method of ethanol delivery (maternal intraperitoneal injection versus intragastric intubation), ethanol dose and mean peak BECs reached. Accepted protocol for scoring the various interactions during play fighting was not observed in the aforementioned study, making comparisons with the current study and others in the literature problematic (Pellis and Pellis, 1987, Pellis and Pellis, 1990). However this contrast in attack frequency is interesting, and many explanations are plausible including different brain areas being affected by ethanol exposure early during gestational development and during the third trimester brain growth spurt. If this ethanol insult during the first trimester of gestation has resulted in widespread cortical and subcortical apoptosis and agenesis, this may have contributed to abnormalities in social functioning that differ from the functional anomalies seen after ethanol exposure during the third trimester. The exact mechanisms of these deficits remain to be elucidated, as Mooney and Varlinskaya (2011) did not investigate neural correlates of the functional change as seen in this thesis.

One anomaly that was not hypothesised in these findings is that the IC group also displayed an increased rate of attack on SC animals. One explanation of this is that the intubation procedure produced some form of stress or trauma-induced changes in the brain of these animals. A literature search did not reveal any studies that have found similar results, however play behaviour studies on alcohol-exposed animals are scarce and therefore it is clear this is an area that needs further investigation. The validity of the intubation procedure may be one explanation for these results, thus it is imperative that these concerns are addressed and investigated through specifically – designed experiments.
The way an animal responds to an attack indicates whether it is interested in continuing to play. Both E4 and IC animals showed a higher rate of supine defence than SC animals, who only showed a 30% chance of going supine when attacked. The SC animals were behaving abnormally here, not the E5 or IC animals, as previous studies have shown the average rate of supine during adolescence to be between 60% and 80% (Pellis and Pellis, 1990). One explanation for this anomaly is that due to the housing arrangements with ethanol-treated animals, SC animals have developed in such a way that cortical and subcortical circuits responsible for normal social responses have been subtly altered. The E5, E4 and IC animals elicited the supine form of defence more than SC animals, indicating the defending animal wanted to continue the play bout when being attacked by animals in these groups, as the supine form of defence has been shown to increase the facilitation of playful interactions (Pellis et al., 1992, Pellis et al., 1997). Again, it is difficult to separate the alcohol-exposed groups from the IC group as they both yielded significant differences than SC animals. However as found in chapters three and four, all alcohol-exposed groups apart from the E4 group in the Acc study showed significant neuronal reductions relative to IC groups in both the CA1 area of the hippocampus and Acc. Therefore even if the intubation procedure is having a detrimental effect on brain function and development, the structural losses are not as severe as the alcohol-exposed animals. Further, as both the hippocampus and Acc are central regions in the proper functioning of social behaviour, the current study may be indicating that the alcohol – exposed animals are exhibiting abnormal behaviour that may not be attributed to the intubation procedure.

Of the few animal models to investigate social behaviour in the rat following neonatal alcohol exposure, it has been found that alcohol-exposed rats exhibit a reduction in aggressive behaviour and also lower testosterone levels after exposure to alcohol throughout development (Lugo et al., 2006). This is in contrast to a prior study by Royalty (1990), which showed an increase in post-pubertal aggressive behaviour after approximately the same period of exposure to alcohol (throughout gestation) (Royalty, 1990). As the current study only used a single day exposure during the third trimester equivalent, it may be of importance for future studies to look into aspects of aggressive behaviour in a social setting – as results have been inconclusive to date.
5.4 Conclusion

The findings from this study pose interesting questions for further research in the field of intubation procedures not only for FASD research, but any developmental-based study paradigms that implement this form of liquid delivery. Multiple differences were found between the ethanol-treated animals and the SC animals, however the IC animals also displayed the same differences. This makes it difficult to attribute the social play interaction disparities to the ethanol exposure during development, and to discount the possible stress related effects of the intubation procedure itself. As the rat brain is still developing at PN6 – neurons are still finalising migration and synaptic stability is still in a state of influence, it is feasible that the hypothalamic-pituitary-adrenal (HPA) axis stress response to intubation has caused lasting changes in brain circuitry and subsequent social functioning.
Chapter 6. PN80-82 Play Behaviour Analysis

6.1 Introduction

As hypothesized in chapter 5, changes in both the frequency of attack and defensive responses were found in adolescent alcohol exposed animals, however not in a dose-dependent manner. If play behaviour is disrupted or abnormal during adolescence, this may lead to the inability to respond to social cues during adulthood in both the rat and human. A human study investigating 2 adults who had suffered prefrontal cortex lesions during early infancy showed a lack of moral and social understanding during adulthood, including the inability to understand future consequences. It was clear that during infancy and adolescence the subtle social and moral cues and nuances had not been acquired, resulting in the inability to interact normally later in life (Anderson et al., 1999). Indeed, rats that have received neonatal mPFC or orbitofrontal cortex lesions display a similar lack of social understanding during the behavioural transition from adolescence to adulthood. Rats with mPFC lesions resort to less complex defensive responses in both adolescence and adulthood, and also attack more, and defend less when attacked (Bell et al., 2009). Rats with orbitofrontal cortex lesions show hyperactivity during play, and fail to modulate their defensive responses with different play partners during adulthood (Pellis et al., 2006).

Few studies have investigated play behaviour in the adult following exposure to alcohol during development and those that have, have used imprecise definitions of play behaviour that make comparisons to existing literature difficult. However, Mooney and Varlinskaya (2011) found that play fighting in young mature rats was reduced in male animals only following a single ethanol binge on gestational day 12. Although indicative of some long-term change, play fighting in their study included pouncing, following and chasing along with the well recognized classifiers, playful nape attack and pinning (supine defence) (Mooney and Varlinskaya, 2011). Although the damage to the prefrontal cortex in the animals in the present study is unlikely to be as severe as the aforementioned ablation studies and it is of a diffuse nature rather than a complete area as in occurs with a lesion it is reasonable to hypothesize that as these animals enter maturity their ability to show normal social function may be impaired. As a level of hyperactivity was shown during adolescence in the form of increases in attack frequency, the normal developmental decrease in attack frequency during adulthood
may not be present. It is also possible that the animals’ response to an attack may be altered due to rewiring of circuits during the adolescent period to compensate for the neuronal loss in the hippocampus and anterior cingulate cortex. In the previous chapter it was suggested that group housing with ethanol animals may have affected the SC animals in some way. Comparison of the interactions between animals in the SC pairs will elucidate whether this is a developmental delay or has resulted in permanent deficits.

6.1.1 Hypothesis

Alcohol – affected animals will not show the age-related reduction in attack frequency or the shift in defensive tactics normally shown in the progression from adolescent to mature play fighting behaviour.

6.1.2 Specific objectives

1. To record play behaviour during adulthood and investigate changes in defensive responses and attack frequencies.
2. To investigate the possibility of functional restitution after neonatal alcohol insult with regard to social play fighting.

6.2 Results

6.2.1 Animals and BEC

The animals used in this older behavioural cohort were the same animals used earlier for PN32-34 play behaviour testing, hence all group sizes (n) were the same (E6 = 11, E5 = 12, E4 = 9, IC = 10, SC = 47). Therefore all treatment and BECs were the same. Animals were kept in the same housing conditions and quadrads until after play testing finished on PN82. All animals survived until this age, and no growths or other physical abnormalities were present.
6.2.2 Attack and Defence

The mean number of attacks in total for each treatment / SC pairing during each ten minute play session was calculated and analysed using a one-way ANOVA, and there was no significant effect of treatment on the mean number of attacks within each pairing ($F = 1.349, P = 0.2529$) (Figure 6.1). This non-significant result is in contrast to that seen in the young animals, where the E6, E5, E4 and IC animals all differed to the SC animals.

Figure 6.1 Total attacks per pairing in a 10-minute period

There was no difference between pairings. Figure indicates mean ± SEM.

The E6/SC grouping does appear to have produced lower mean numbers of attacks, however was not significant. The mean attack values displayed in this figure were approximately 1/3 that of the younger PN32-34 animals, indicating a large reduction in number of bouts during each 10 minute period during maturity.
The mean number of attacks in ten minutes elicited by the treatment groups on SC was also analysed (Figure 6.2). No significant differences were found between any groups, although again the E6 group showed less attacks than all other groups. LMM analysis confirmed with this finding with no significant group differences in the rate of attack on SC animals (Table 6.1).

**Figure 6.2** Shows the mean attacks by each treatment group on SC

This figure is similar to Figure 6.1, showing no significant differences (P > 0.05) between groups however the E6 group does appear to be making less attacks on SC animals. Data represent mean ± SEM.
Table 6.1 LMM analysis of number of attacks per 10-minute period by treatment group, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>-0.18</td>
<td>2.42</td>
<td>0.942</td>
<td>(-4.91 - 4.56)</td>
</tr>
<tr>
<td>E4</td>
<td>0.84</td>
<td>2.49</td>
<td>0.736</td>
<td>(-4.04 - 5.72)</td>
</tr>
<tr>
<td>E5</td>
<td>-0.42</td>
<td>2.34</td>
<td>0.858</td>
<td>(-5.00 - 4.16)</td>
</tr>
<tr>
<td>E6</td>
<td>-2.67</td>
<td>2.34</td>
<td>0.253</td>
<td>(-7.25 - 1.91)</td>
</tr>
</tbody>
</table>

There was also no significant differences between groups for attacks made by the SC group on all other treatment groups (P > 0.05), although again it appeared that the frequency of attacks made on the E6 group may have differed in some way (Figure 6.3). LMM analysis confirmed this lack of difference however there does appear to be a grouping of behaviour in the E6 and E4 groups showing similar reductions in the number of attacks inflicted with a trend towards less attacks being made on the E6 and E4 groups – showing an average difference of 3.3 and 3.5 less attacks respectively when compared to SC (P = 0.098, 0.1 respectively) (Table 6.2).
Figure 6.3 Mean attacks made by SC on other treatment groups

A one-way ANOVA did not reveal any differences between groups, however less attacks were made on the E6 animals. P > 0.05.

Table 6.2 LMM analysis of number of attacks on each treatment group by SC animals, relative to other SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>-0.37</td>
<td>2.10</td>
<td>0.862</td>
<td>(-4.48 - 3.75)</td>
</tr>
<tr>
<td>E4</td>
<td>-3.45</td>
<td>2.09</td>
<td>0.1</td>
<td>(-7.55 - 0.66)</td>
</tr>
<tr>
<td>E5</td>
<td>-1.22</td>
<td>2.00</td>
<td>0.541</td>
<td>(-5.15 - 2.70)</td>
</tr>
<tr>
<td>E6</td>
<td>-3.31</td>
<td>2</td>
<td>0.098</td>
<td>(-7.23 - 0.62)</td>
</tr>
</tbody>
</table>
The probability of showing some form of defence to an attack did not reveal any differences, as with the adolescent animals (F = 0.701, P = 0.593) (Figure 6.4). LMM analysis also did not find any differences in the probability of defence between pairings.

![Figure 6.4 Probability of defence](image_url)

No differences were found between any groups following both a one-way ANOVA and LMM analysis. All groups showed high rates of defence. Figure shows mean probability of defence after attack by SC animal. Note Y-axis scale starting at 80%.

The probability of supine, partial or evasive defence when each animal of a pairing was either defender or attacker, was analysed as for the adolescent animals. One-way ANOVA showed that probability of supine (F = 0.420, P = 0.795) and partial (F = 1.932, P = 0.110) defence did not differ between groups when the treated animals were defending against attack by an SC animal (Figures 6.5A, 6.5B). The LMM analysis however did not concur with the ANOVA, indicating that E6 animals show a significant reduction (coefficient of 1.9) in the frequency of adopting a supine defence to attack by an SC animal relative to SC animals (P < 0.05) (Table 6.3).
**Table 6.3** LMM analysis of supine defence per 10-minute period by treatment group, when compared with SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>-0.35</td>
<td>0.97</td>
<td>0.717</td>
<td>(-2.25 - 1.55)</td>
</tr>
<tr>
<td>E4</td>
<td>-1.44</td>
<td>0.93</td>
<td>0.139</td>
<td>(-3.35 - 0.47)</td>
</tr>
<tr>
<td>E5</td>
<td>-1.20</td>
<td>0.93</td>
<td>0.198</td>
<td>(-3.02 - 0.63)</td>
</tr>
<tr>
<td>E6</td>
<td>-1.91</td>
<td>0.97</td>
<td>0.041</td>
<td>(-3.73 - 0.08)</td>
</tr>
</tbody>
</table>

The probability of supine defence being elicited in the other animal following an attack by an animal from a treatment group was different between treatment groups (P < 0.05). However the one-way ANOVA only just reached the level of significance (P < 0.05) and subsequent post-hoc testing using the Newman-Keuls adjustment did not show any differences between groups. However it appears in Figure 6.6A that the IC animals appear to be eliciting the supine defence more than SC animals. LMM analysis also did not show any significant differences between the groups (Table 6.4).

**Table 6.4** LMM analysis of supine defence elicited in the SC play partner, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>0.75</td>
<td>1.09</td>
<td>0.488</td>
<td>(-1.38 - 2.89)</td>
</tr>
<tr>
<td>E4</td>
<td>0.13</td>
<td>1.17</td>
<td>0.914</td>
<td>(-2.17 - 2.43)</td>
</tr>
<tr>
<td>E5</td>
<td>0.03</td>
<td>1.07</td>
<td>0.977</td>
<td>(-2.07 - 2.14)</td>
</tr>
<tr>
<td>E6</td>
<td>-0.47</td>
<td>1.07</td>
<td>0.658</td>
<td>(-2.57 - 1.62)</td>
</tr>
</tbody>
</table>
Although the one-way ANOVA did not detect any between group differences, LMM analysis revealed values for the frequency of partial defence from SC attack for E4 (P = 0.087) and E6 (P = 0.064) groups that suggest a trend towards a reduced use of the partial defence strategy (Table 6.5).

Table 6.5 LMM analysis of partial defence by treatment group following SC attack, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>-0.46</td>
<td>0.81</td>
<td>0.570</td>
<td>(-2.05 - 1.13)</td>
</tr>
<tr>
<td>E4</td>
<td>-1.42</td>
<td>0.83</td>
<td>0.087</td>
<td>(-3.04 - 0.21)</td>
</tr>
<tr>
<td>E5</td>
<td>-0.96</td>
<td>0.77</td>
<td>0.216</td>
<td>(-2.48 - 0.56)</td>
</tr>
<tr>
<td>E6</td>
<td>-1.43</td>
<td>0.77</td>
<td>0.064</td>
<td>(-2.94 - 0.08)</td>
</tr>
</tbody>
</table>
Figure 6.5 Probability of various forms of defence in response to an attack by an SC play partner

Figures A, B and C show the probability of supine, partial and evasive defence respectively during adulthood by treatment group, measured as a percentage. E6 group used the evasive defence more than SC animals (P < 0.05).
Probability of partial defence elicited by IC and E6 animals in an SC play partner appeared to be less than SC animals, and analysis with one-way ANOVA revealed a significant effect of treatment (P < 0.05) (Figure 6.6B). However as for the supine analysis, post-hoc testing (Newman Keuls) failed to produce any individual group differences. LMM analysis was consistent with this finding, not revealing any group differences (Table 6.6).

**Table 6.6** LMM analysis of partial defence elicited in an SC play partner by treatment group animals, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>-1.25</td>
<td>1.18</td>
<td>0.289</td>
<td>(-3.57, 1.06)</td>
</tr>
<tr>
<td>E4</td>
<td>0.01</td>
<td>1.24</td>
<td>0.991</td>
<td>(-2.42, 2.45)</td>
</tr>
<tr>
<td>E5</td>
<td>-0.73</td>
<td>1.16</td>
<td>0.526</td>
<td>(-3.00, 1.53)</td>
</tr>
<tr>
<td>E6</td>
<td>-1.39</td>
<td>1.16</td>
<td>0.23</td>
<td>(-3.65, 0.88)</td>
</tr>
</tbody>
</table>

The probability of an evasive defence in treated animals following an attack by an SC animal differed significantly between groups (F = 2.993, P < 0.05) (Figure 6.5C). Post-hoc analysis using the Newman-Keuls adjustment showed the E6 group used the evasive defence more than SC animals (P < 0.05). However LMM analysis did not reveal any differences (Table 6.7) – a finding in contrast to the ANOVA.

**Table 6.7** LMM analysis of evasive defence by treatment group in response to SC attack, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>0.18</td>
<td>0.84</td>
<td>0.832</td>
<td>(-1.46, 1.82)</td>
</tr>
<tr>
<td>E4</td>
<td>-0.57</td>
<td>0.84</td>
<td>0.498</td>
<td>(-2.22, 1.08)</td>
</tr>
<tr>
<td>E5</td>
<td>0.94</td>
<td>0.79</td>
<td>0.238</td>
<td>(-0.62, 2.49)</td>
</tr>
<tr>
<td>E6</td>
<td>0.24</td>
<td>0.79</td>
<td>0.767</td>
<td>(-1.32, 1.79)</td>
</tr>
</tbody>
</table>
There were no differences in the probability of evasive defence elicited in an SC play partner by treated animals, as shown by ANOVA (F = 1.157, P = 0.334) (Figure 6.6C) and LMM analysis (Table 6.8).

Table 6.8 LMM analysis of evasive defence elicited in an SC play partner, relative to SC animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>0.52</td>
<td>0.94</td>
<td>0.583</td>
<td>(-1.33 - 2.36)</td>
</tr>
<tr>
<td>E4</td>
<td>0.65</td>
<td>0.95</td>
<td>0.496</td>
<td>(-1.22 - 2.51)</td>
</tr>
<tr>
<td>E5</td>
<td>-0.43</td>
<td>0.89</td>
<td>0.631</td>
<td>(-2.17 - 1.31)</td>
</tr>
<tr>
<td>E6</td>
<td>-0.69</td>
<td>0.89</td>
<td>0.435</td>
<td>(-2.43 - 1.05)</td>
</tr>
</tbody>
</table>

Probabilities of various defence parameters elicited in the SC play counterpart, by each treatment group, did not reveal any inter-group differences (Figure 6.6). Although a one-way ANOVA revealed a significant effect of treatment in partial and supine defence elicited in the SC play partner, post-hoc analysis did not reveal significant differences.
Both figures A and B revealed significant treatment effects of the probability of supine and partial defence elicited, however post-hoc testing did not show any individual differences. Probability of evasive defence elicited (figure C) was not significantly different between treatment groups. Group in brackets indicates defending animal (SC).
The differences between attack and defence parameters for the animals during adolescence (Chapter 5) and then maturity (current chapter) were also analysed. Two-way ANOVA was used to analyse the interaction between treatment group and age for each attacking and defensive parameter.

A significant treatment by age interaction was found for attack frequency (F = 3.49, P < 0.01), along with a treatment (F = 2.93, P < 0.05) and age effect (F = 91.93, P < 0.001) (Figure 6.7). This confirms that some treatment groups showed significant decreases in the frequency of attack between adolescence and maturity. Interestingly, all groups apart from SC animals showed a significant decrease in attack frequency between adolescence and maturity following a one-way ANOVA and Newman-Keuls test (P < 0.001).

Figure 6.7 Attack frequency during adolescence and adulthood

All groups apart from SC animals showed a decrease in the frequency of attack between PN 32 and PN80 (P < 0.001).
Analysis of probability of defence revealed no interaction between treatment and age ($F = 0.82, P = 0.515$), no effect of treatment ($F = 1.05, P = 0.381$) and no effect of age ($F = 2.98, P = 0.086$) (Figure 6.8A). Rates of defence remained constant in both age brackets and between ages, with no group differences. This implies that all animals showed the same amount of motivation to defend following an attack.

The probability of supine defence ($F = 3.99, P < 0.01$) showed a treatment by age interaction (Figure 6.8B). Treatment was also a significant source of variation ($F = 3.99, P < 0.01$) along with age ($F = 39.32, P < 0.001$). This indicates the supine form of defence was used significantly more during adolescence than when mature for some groups. Post-hoc testing revealed that only E4 and IC animals showed reduced rates of supine defence between adolescence and maturity ($P < 0.05, P < 0.001$ respectively).

The probability of partial defence did not show a significant interaction between treatment group and age ($F = 0.69, P = 0.597$), however there was a significant effect of treatment ($F = 3.40, P < 0.05$) (Figure 6.8C). The effect of age on the probability of responding to an attack with a partial defence was approaching significance ($F = 3.71, P = 0.055$). That the probability of the partial form of defence did not vary between the adolescent and mature old animals was an unexpected finding.

There was a significant increase in the probability of mounting an evasive defence in mature animals in contrast to adolescence animals as indicated by the significant interaction between treatment and age ($F = 2.56, P < 0.05$) (Figure 6.8D). Treatment alone was also a significant source of variance ($F = 2.73, P < 0.05$), along with age ($F = 45.28, P < 0.001$). This indicates that as the animals became mature, the evasive form of defence became preferred, particularly over the supine response. Post-hoc testing showed that evasive defence increased between adolescence and maturity in the E6 group ($P < 0.01$), the E5 group ($P < 0.05$) and the IC group ($P < 0.01$). Both E4 and SC animals did not show any age related probabilities in the use of evasive defence.
Figure 6.8 Probability of various forms of defence during adolescence and maturity

These figures show the relative probability of any form of defence (A), supine defence (B), partial defence (C) and evasive defence (D) during PN32-34 (adolescence) and PN80-82 (maturity).
6.3 Discussion

The current study investigated the effect of a single ethanol binge on PN6 on play behaviour in the adult rat. The age-related shifts in attack and defence responses were also analysed to investigate the possibility of functional recovery and brain repair after an insult of this magnitude.

No differences were found between groups in frequency of attack over the ten-minute play-fighting period, although the E6 animals appeared to attack slightly less than the other groups. This is interesting, as during adolescence all treatment groups and their SC counterparts had a higher number of attacks than the SC/SC group. It appears that some level of recovery was evident as the animals matured. The mean rates of attack per 10-minute session seen in this study were similar to previous play behaviour studies, however a degree of variability is apparent between studies, even within strains and ages. Animals from all groups attacked between five and ten times on average – a figure found in Long-Evans rats aged 90-100 days old (Pellis et al., 2006) and 75 days old (Pellis and Pellis, 1990), however other studies using similar parameters have found the mean number of attacks per animal to be approximately 20 per 10-minute period (Smith et al., 1997, Kamitakahara et al., 2007). Both of the aforementioned studies also only used male rats, which typically show a higher rate of attack than females throughout development (Pellis et al., 1997). Therefore, when compared to the literature the animals from all groups appear to be showing normal rates of attack – and thus one explanation is the ethanol-exposed and IC animals may have displayed signs of recovery of social function during maturity. It is possible that circuits integral for appropriate levels of attack have been rewired or have undergone plastic changes between the adolescent and adult period to allow better adaptation of social behaviour.

Probability of some form of defence in response to attack (i.e. not “no response” – where the animal does not respond or evade at all) was consistently high across all groups and no differences were found in either the young or old animals. This is consistent with previous studies, where animals typically defend over 80% of the time (Pellis and Pellis, 1990, Smith et al., 1997). This indicates that all animals, even the ethanol-exposed groups are reacting to attacks in the same manner. Therefore loss of somatosensory function or motor abilities is unlikely, as both of these entities require proper functioning for an animal to be effective in defence. This is diverse to findings
from Laurence and colleagues (2007), who showed that animals exposed to ethanol throughout gestation exhibit deficits in processing somatosensory cues manifest as an increase in frequency of supine defence (Lawrence et al., 2008). Lawrence and colleagues exposed these animals to ethanol throughout gestation – a much larger insult than the single binge used in the current study, therefore it is possible that a threshold effect might be evident for changes in somatosensory areas to occur.

No differences were found between groups in probability of supine defence. This indicates all animals were showing similar defensive patterns, however the actual percentage values were very low when compared to typical probabilities in the literature. Animals aged 70-75 days old typically show a 40-70% chance of going supine when attacked (Pellis and Pellis, 1990), and a 30% chance of going supine at 90-100 days of age (Kamitakahara et al., 2007), compared with 20-25% in the current study. As all groups displayed similar levels of supine defence, it is possible that the ethanol-exposed animals have had an influential effect on the behavioural response in the control animals due to combined housing throughout development and testing. This is similar to a study by Deak and Panksepp (2006), where scopolamine treated animals induced an increase in play solicitation in their control partner (Deak and Panksepp, 2006). The SC animals may not have received a normal development as they were housed with putatively abnormal treatment animals, and this may have resulted in their behaviour being altered to adjust to the abnormal social situation. It may be interesting for future studies to incorporate a third control group into testing, where housing SC animals with other SC animals may produce different results due to this possibility.

Due to the low probability of supine defence, the probability of evasive defence was abnormally high (30-50%). Animals aged 90-100 days old evade approximately 20% of the time (Pellis and Pellis, 1990, Kamitakahara et al., 2007). E6 animals showed a significantly higher probability of evading an attack than SC animals, and this suggests these animals are trying to terminate the play bout or are perhaps more intimidated by the SC animals and were looking to end further contact. To date only two studies have investigated the effects of perinatal ethanol exposure on play behaviour, and both of these have had very different ethanol doses, timing of administration and have analysed different aspects of play behaviour – making it difficult to make direct comparisons with the present study. Meyer and Riley (1986) found that following ethanol exposure throughout gestation, male animals do not show the typical higher rate of play than
females, and female animals exposed to ethanol exhibit masculinized behaviour (increased rate of play) (Meyer and Riley, 1986). This suggests that ethanol exposure during gestation alters the hormonal environment during development, and thus the later androgen levels in adolescent animals. Testing was conducted during the light cycle, whereas the current study was conducted during the dark cycle and hence animals would show heightened levels of activity. Interestingly, Lawrence and colleagues (2007) did not find any differences in rates of attack in animals that were exposed to ethanol throughout gestation in a similar regime to the study by Meyer and Riley (1986) (Lawrence et al., 2008). It is however clear that perinatal ethanol exposure is having some form of effect on play behaviour, and more work is needed to fully elucidate the sometimes subtle, but important differences.

An important finding of the current study was that the SC animals were the only group not to show the typical decrease in attack frequency between adolescence and maturity. All other groups, including IC animals showed a significant decrease in the number of attacks in each 10-minute session. No studies to date have looked at the effect of perinatal ethanol on play fighting during adulthood; therefore comparisons can only be made with control data in the literature. The typical rate of attack for control animals during adolescence (PN30) is approximately 30/animal/10 minutes (Pellis and Pellis, 1990). SC animals in the current study showed only 17 attacks per ten minutes on average, well below all other groups. Subsequently, as the initial rate of attack during adolescence was already very low, the rate during adulthood did not change. The adolescent rate was abnormally low, which suggests that these SC animals were behaving differently due to their exposure to treated animals during development, as all animals were housed in quadrads with two SC animals and two treatment animals per cage. As all other groups displayed much higher rates of attack than SC animals it is reasonable to assume these animals were either overly aggressive or hyperactive. As a consequence, SC animals may have adapted to this hyperactivity by reducing their rates of attack to compensate for the extra activity while being attacked, thereby altering their own normal social development. Further, the SC animals were the only group to fail to reveal a shift in any of the attacking or defensive parameters measured between adolescence and adulthood.

Probability of supine defence only decreased from PN32 to PN80 in the E4 and IC groups, indicating abnormal defensive patterns in all other groups. Typically, the rate of
supine defence during adolescence should be around 60-70%, however SC, E5 and E6 groups were only going supine 30-40% of the time. It is interesting that these groups, which failed to show a decline in supine defence with age were both high-dose ethanol groups, and the SC control group. One explanation for this is that the high dose ethanol animals were showing a higher level of aggressiveness and hence displayed higher levels of partial facing defence or evasive behaviour – unwilling to rotate to the more vulnerable supine position. The SC animals may have altered their defensive behaviour during juvenile development, and similar to the high-dose ethanol animals have reduced their rate of supine defence, but due to different reasons. Evading from a constantly attacking animal may be a more effective form of defence than supine defence, which gives the pinned animal limited escape options.

Evasive defence typically remains constant between adolescence and adulthood at around 15-20% (Pellis and Pellis, 1990), and the E4 and SC animals in this study were the only groups to show constant rates of evasive defence between adolescence and adulthood. All other groups exhibited increases in the probability of evasive defence during adulthood. Therefore the E4 and SC groups displayed normal behaviour with regard to evasive defence, however E6, E5 and IC groups displayed an abnormal rise in evasive defence during adulthood. This may be due to hyperactivity, or changes in their ability to respond to and reciprocate attacks. The high rate of attack frequency during adolescence may have manifest as a high rate of evasive defence in adulthood, suggesting possible plastic changes in brain circuits involved with regulating attack and defensive responses. These abnormal defensive responses seen in the current study may be the result of abnormal levels of anxiety or fear responses in either ethanol-exposed animals or in the control groups as a result of housing with the ethanol treated animals.

6.4 Conclusion

After a single binge ethanol insult on PN6, it appears that treatment animals still possess the full repertoire of attack and defensive responses during adulthood, although some of these responses are abnormal in their frequency and relative change from adolescence. Low rates of supine and high rates of evasive defence in ethanol-exposed animals in adulthood indicate a lack of reciprocity – and in turn termination of play bouts. These differences are however very subtle, and it seems that relative to adolescence the ethanol-exposed animals show much more typical social behaviour as adults. It is possible the cell loss in the Acc and CA1 area of the hippocampus contributed to the
high frequency of attack in adolescence, however some restitution of function has occurred during adulthood. It is also likely that numerous other brain areas have been damaged by the alcohol insult, and future studies will need to further investigate this structural-functional relationship before causative links can be made.

Chapter 7. Elevated T-Maze

7.1 Introduction

Following the investigation of play behaviour, it is clear that adolescent alcohol-exposed animals exhibit hyperactivity, manifest as an increased number of attacks per play session. One explanation for this is that the ethanol-exposed animals were displaying anxious behaviour, either due to the new environment or confrontation with another animal. This hyperactivity was diminished during adulthood, and this also raises questions about the possible recovery of social behaviour and/or reduction in anxiety during maturity.

Children and adolescents with FASD typically show hyperactivity, attention deficits, and lack of response inhibition (impulsivity) (Streissguth, Streissguth et al., 1983, Streissguth et al., 1985, Streissguth et al., 1986, Nash et al., 2011, Rasmussen et al., 2011). Rats exposed to prenatal alcohol of some degree exhibit diminished shock avoidance learning (Bond and Giusto, 1977) and 2-way avoidance conditioning (Bond and DiGiusto, 1978). A lack of response inhibition also correlates with the human data, indicating a lack of emotional control in certain situations (Riley et al., 1979, Gallo and Weinberg, 1982).

Numerous animal model studies have anxiety-like behaviour following prenatal exposure to ethanol (Weinberg, 1996, Dursun et al., 2006, Zhou et al., 2010). Exposure to high levels of alcohol during gestation resulted in decreased entry into the centre of the open field and decreased rearing in alcohol-exposed offspring during early adulthood, both indicators of increased anxiety (Zhou et al., 2010). Rats exposed to ethanol during the first 2 trimesters also show hyperactivity and anxiogenic behaviour in the elevated plus-maze (EPM), the precursor to the elevated T-maze. Both ethanol-exposed males and females showed higher levels of exploratory behaviour when placed in the EPM, however only females exhibited an increased fear response and higher
corticosterone levels (Osborn et al., 1998). One advantage of using the ETM over the EPM is that it has the ability to measure 2 types of anxiety–related behaviours, generalized anxiety disorder (GAD) and panic disorder (Viana et al., 1994, Zangrossi and Graeff, 1997). This can be important when thinking about possible therapeutic interventions as different drugs are used to treat these disorders.

The ETM relies on spontaneous behaviour, therefore does not require prior training, food deprivation or exposure to noxious stimuli – making it an ideal test to be preceded or proceeded by other behavioural testing. It is also important to note that without these potential confounds that may affect experimental groups differently to control, data produced may be more applicable to a human setting. The impact on anxiety after a single binge-exposure to ethanol using behavioural testing on the ETM has never been investigated. This is entirely relevant to the results found in both the stereological and play behaviour studies examined in chapters 3-6, as the hippocampal-Acc-amygdala circuit is an integral part of anxiety-related pathologies, and the hyperactivity and altered defensive responses seen in the play testing may be indicative of an underlying anxiety or fear related response.

7.1.1 Hypothesis

A single alcohol exposure on postnatal day 6 results in an increase in generalized anxiety in adulthood.

7.1.2 Specific objectives

To obtain data from both inhibitory avoidance and one-way escape paradigms, and assess differences or similarities of generalized anxiety and panic levels between control and alcohol-treated animals.

7.2 Results

7.2.1 Inhibitory Avoidance

Group numbers (n) were; n (E6) = 8, n (E5) = 10, n (E4) = 6, n (IC) = 8, n (SC) = 7. The first measure analysed was the innate fear of open spaces that rodents possess, and is measured by the acquisition of the inhibitory avoidance test. Proper inhibitory avoidance acquisition is displayed when the animal takes progressively longer to leave
the enclosed arm from baseline performance to trial 1 and 2. If the animal spends relatively more or less time in the enclosed arm, this indicates an anxiogenic or anxiolytic effect respectively.

As indicated by Figure 7.1, the alcohol-exposed animals did not show gradual increases in time spent in the enclosed arm, and a rather erratic performance is evident over the 3 trials.

**Figure 7.1** Inhibitory Avoidance over 3 trials

This figure represents the time taken for animals to leave the enclosed arm during the 3 trials of inhibitory avoidance. Variability was large in some trials due to some animals taking much longer to exit the enclosed arm. Data represent mean ± SEM. Numbers 1, 2 and 3 on the X-axis represent each of the three successive inhibitory avoidance trials.
Further to the previous Figure 7.1, Figure 7.2 below shows the relative latencies in each of the three inhibitory avoidance trials for each treatment group. The E6 group appeared to have a longer latency to leave the enclosed arm in the baseline trial, however likely due to large variability within the group this was not significantly more than the control groups.

Figure 7.2 Inhibitory Avoidance by treatment and trial number

A two-way ANOVA with treatment and trial number as factors did not reveal a significant interaction between treatment and trial number \( (F = 0.56, P = 0.8084) \). Treatment group did not affect the result \( (F = 1.51, P = 0.8084) \), nor did trial number \( (F = 0.97, P = 0.3841) \).
As the variability was very different between the groups, data was log transformed, which reduced variability between treatment groups allowing accurate one-way ANOVAs to be carried out for each of the 3 trials (Figure 7.3).

**Figure 7.3** Baseline trial of Inhibitory Avoidance by Treatment

Trial 1 of inhibitory avoidance acquisition shows that alcohol exposed animals tended to remain longer in the enclosed arm than controls. However a one-way ANOVA showed this was not a significant effect (P = 0.0581). Data has been log transformed to ensure equal variability between groups. Data represent mean ± SEM.

Avoidance trials 1 and 2 did not reveal any significant differences in acquisition between treatment groups (trial 1 F = 1.308, P = 0.2867 and trial 2 F = 0.2845, P = 0.8860 respectively). Subsequently, a ratio of time difference between trials was calculated using the formula (trial 1 / trial 2 * 100) to analyse the initial rate of acquisition of learned avoidance behaviour.
The E6 group took significantly less time than both IC and SC animals to leave the enclosed arm in trial 2 than in trial 1, as measured by the ratio \( F = 3.14, P < 0.05 \) (Figure 7.4).

**Figure 7.4** Trial 1/Trial 2 ratio as a percentage

A one-way ANOVA showed a significant effect of treatment on the ratio between trial 1 and trial 2 \( F = 3.14, P < 0.05 \). Post-hoc analysis revealed the E6 group had a significantly higher T1/T2 ratio than both IC and SC animals. Data represent mean ± SEM.

One-way ANOVA analysis of the ratio between baseline and trial 2 \( F = 1.17, P = 0.34 \), and trials 1 and 2 \( F = 0.523, P = 0.72 \) did not find any significant interaction between treatment and ratio.

These results pose an interesting picture of how the E6 group reacted in the initial stages of inhibitory avoidance acquisition. Compared to both IC and SC groups, E6 animals spent significantly more time in the enclosed arm in trial 1 compared to trial 2.
7.2.2 One-way escape

Animal group numbers (n) were as for inhibitory avoidance testing. Following the 3 inhibitory avoidance trials, animals were subjected to 3 successive escape trials. Classically this test can be linked to panic disorder based on innate fear (Figure 7.5).

**Figure 7.5** One-way escape over 3 trials

This figure shows the time taken across 3 consecutive trials to escape from the extreme end of the open arm. Data represent mean ± SEM.
The only difference found between treatment groups in the one-way escape trials was during the baseline trial. The E6 group was found to take significantly less time to escape from the open arm than the E4 animals ($F = 2.77, P < 0.05$) – an anticipated finding, as the E6 animals spent longer in the enclosed arm than E4 animals before exiting during the inhibitory avoidance testing (Figure 7.6).

**Figure 7.6** Baseline trial of one-way escape by Treatment

This figure shows log-transformed data of the time to escape out of the open platform. The E6 group was found to take significantly less time to escape than the E4 group ($* P < 0.05$). Data represent mean ± SEM.

A one-way ANOVA of escape trials 1 and 2 did not show any differences in time to escape attributed to treatment group (trial 1 $F = 1.496, P = 0.2251$, trial 2 $F = 0.3386, P = 0.85$) (Figure 7.7).
A two way ANOVA with treatment group and trial number as factors did not reveal a significant interaction ($F = 1.10, P = 0.3684$) indicating that the effect of treatment does not vary between trials. Treatment group did not affect the result ($F = 1.22, P = 0.3086$), nor did trial number ($F = 1.00, P = 0.3702$). A Newman-Keuls post-hoc test did not show any differences between individual treatment groups. Data represent mean ± SEM.

Figure 7.7 One-way escape by treatment and trial number
As with the inhibitory avoidance analysis, a ratio of time difference between trials was calculated using the formula (trial 1 / trial 2 * 100) to analyse rates of acquisition from a different perspective. A one-way ANOVA showed a significant effect of treatment (F = 3.674, P < 0.05). Post-hoc analysis showed E5 animals had a significantly higher T1/T2 ratio than E6, IC and SC animals (P < 0.05) (Figure 7.8).

**Figure 7.8** Trial 1/Trial 2 ratio as a percentage

This figure shows the relative time in percentage of the first trial that each group took to escape in the second trial. Percentages over 100 indicate trial 2 took less time than trial 1. * indicate means are significantly different (P < 0.05). Data represent mean ± SEM.
7.3 Discussion

The current study hypothesized that changes in anxiety-related behaviour, as measured using the ETM, would reveal anxiogenic behaviour in animals exposed to alcohol on PN6. In some ways this was accurate, however some interesting conflicting results were found in relation to the range of values recorded with reference to typical values in the literature.

The first test the animals were subjected to was the inhibitory avoidance paradigm. This test is unconditioned – insofar that the animal has not previously been subjected to the closed arm. A sign that proper acquisition has been obtained is when the latency to leave the enclosed arm increases through successive trials. Rats have an innate fear of open spaces, and this, together with a lack of walls to allow thigmotaxic orientation results in the animal preferring to occupy the enclosed arm. Although not significant, all three alcohol exposed groups showed a rather erratic sequence of latencies to leave the enclosed arm over successive trials when compared to both control groups, which displayed the normal increase in latency over the three trials. This may infer that the ethanol exposed animals either forgot after the first trial that the open arm was not a desirable place to be, or they simply did not have the same amount of aversion to the open arms as the control animals. This supports animal (Riley et al., 1979, Gallo and Weinberg, 1982) and human evidence (Streissguth et al., 1985, Olson et al., 1992, Salmon, 2008) that rats prenatally exposed to ethanol and children with FASD show a lack of response inhibition when placed in certain situations. Interestingly, the two-way ANOVA showed that none of the treatment groups acquired the inhibitory avoidance test, as trial number did not affect the outcome. During trial 1 and 2 of inhibitory avoidance testing, one animal in the E6 group took a much longer amount of time to venture out into the open arm than the other animals, thus altering data averages and variances somewhat. This animal may have been more affected by the ethanol than the other animals, causing this increased fear response and subsequent long latencies (Figure 7.1).

Long-Evans rats are not typically used in behavioural testing, as Wistar or Sprague-Dawley rats are considered the standard for pharmacological testing – the reason behind most ETM testing. It is possible this strain of rat – which is very representative of the typical wild rat, is less prone to anxiety – related behaviour as the latency to exit the
closed arm was very small relative to other studies investigating prenatal stress and anxiety (Estanislau and Morato, 2005) and hippocampal lesions and anxiety (Trivedi and Coover, 2004). Perhaps these animals needed more trials to reach proper acquisition, as the experience of venturing into the open arms did not appear to be very aversive, as shown by the relatively small latencies.

Although not quite significant (P = 0.0581), the inhibitory avoidance baseline trial revealed a propensity for the ethanol-exposed animals to stay in the enclosed arm for longer before venturing out into the open arms, and supports evidence that neonatal ethanol exposure increases anxiety levels in both rats (Dursun et al., 2006) and humans (Hellemans et al., 2010). This indicates a level of anxiety to this novel environment, however this anxious behaviour was not present during trials two or three – as shown by the ratio figures. In fact the E6 animals showed a greater reduction in latency to leave the closed arm in trial two than both control groups indicating a rather large change in anxiety levels. This is unusual, and suggests that these E6 animals were initially very scared to enter the aversive open arms, but once they did and realised it did not pose a threat, they were more interested in exploring the maze in subsequent trials than retreating from a possible threat.

An interesting anomaly in the one-way escape testing was that the E6 animals took significantly less time to escape out of the open arm in the baseline trial than the E4 animals. The baseline inhibitory avoidance trial indicated a higher level of anxiety in the ethanol-exposed relative to control groups. This result implies that only the E6 animals are showing an abnormal level of panic when placed in this conditioned open arm situation, but only relative to E4 animals. It is possible that the higher dose of ethanol has induced anxiogenic behaviour, while the lower 4.5g/kg dose is having the opposite, or no observable effect. Another unexpected result was that the E5 animals took significantly less time to escape from the open arm in trial 2 relative to baseline trial than both control groups, and the E6 group. Therefore the E5 animals exhibited a larger panic response when placed onto the open arm in the second trial. It is difficult to decipher the meaning of this as the latencies varied from both the control and E6 animals. However it appears the E6 animals were acting normally relative to controls during trial 2, therefore it can be concluded that the E5 animals had some sort of delayed reaction to the experience of being on the open arm.
Due to many animals either being deceased or discounted from this study due to tumours and other illness, the numbers in each group were between six and ten and this may have impacted on statistical power. It would definitely be of interest for future studies in this area to use the ETM to assess the role of anxiety in the altered behavioural phenotypes of alcohol-exposed animals. It appears in these early animal studies and human reports that anxiety plays a part in the altered psycho-social elements of the FASD, and many simple therapeutic interventions exist for treatment of these disorders which is promising as many aspects of FASD still elude researchers as to a remedy.

7.4 Conclusion

It is evident that the high-dose ethanol-exposed animals used in this study of anxiety displayed abnormal behaviour on both the inhibitory avoidance and one-way escape testing, relative to control groups. This may mean that just one exposure to ethanol during development can change the way threatening stimuli are perceived and processed. The E6 animals essentially showed a lack of the appropriate fear response and acquisition of learned behaviour to a threatening environmental situation. This lack of inhibition may be relevant in social and environmental situations where judgement and insight, or lack thereof could be the difference between overtaking that car safely and another road toll statistic. This investigation into anxiety also revealed important implications for the aforementioned disparity between IC animals and SC animals in the adolescent play behaviour testing. If the intubation procedure caused animals to function differently during play fighting, this was not translated to innate or acquired anxiogenic situations. Therefore it is possible that the brain circuit responsible for anxiety and response inhibition is affected to a greater extent by the ethanol exposure than the prefrontal cortical circuits involved in play behaviour. It may also be that only responses requiring more demanding cognitive processing are susceptible to the neuronal loss seen in chapters 1 and 2, as stressful situations such as the ETM are needed to elicit the true extent of the structural loss.
Chapter 8. General Discussion

This study aimed to investigate the effect of a single ethanol binge on postnatal day 6 on permanent cell loss in the hippocampus and anterior cingulate cortex. The behavioural manifestations of these specific structural deficits were then established through social play and anxiety behavioural analysis.

8.1 Ethanol-induced permanent cell loss in the hippocampus and anterior cingulate cortex after a single binge on PN6

The finding of significant importance from this study was that a single-day binge exposure to ethanol results in extensive cell death in both the hippocampal CA1 area and the Acc in a dose-dependent manner. Previous work has shown cellular losses in the cerebellum following a two-day binge (Thomas et al., 1998a), and single-day binge (Pauli et al., 1995a). However this is the first study to document substantial losses in these important brain regions for learning and memory, social and cognitive reasoning and executive functioning following a single binge exposure to ethanol. This is important, as human data suggests mothers who do drink during pregnancy do not drink on multiple, successive days but rather in high amounts on single days (Watson and McDonald, 1999b). Therefore if these mothers have a binge drinking session during the third trimester, it is likely that significant irreversible cell loss will have occurred in the hippocampus and Acc of the developing foetus. Apoptosis has been shown to begin to occur between 3 and 4 hours after ethanol exposure, therefore intervention is needed during or soon after the drinking session - as once cell death has been initiated amelioration is less likely to be effective (Fowler, 2007).

E6 and E5 ethanol-exposed groups showed growth deficits following ethanol administration up until PN14, and from this point on weights were similar across groups. This shows that a single ethanol binge in the third trimester will not result in permanent growth deficits, and subsequently children who have been exposed to a similar insult may not have the prenatal and postnatal growth deficits that are useful in establishing that they have FASD. The lack of craniofacial abnormalities and severe mental retardation would also make a diagnosis improbable. As physical growth has
not been permanently disrupted, but neuronal survival has, this suggests the central nervous system is particularly sensitive to the effects of ethanol and as this study has identified, only a single binge during the third trimester is enough to induce apoptosis on a large scale. Gross cerebellar weight was permanently reduced, and this concurs with previous work by Goodlett and colleagues (1989) which showed a permanent reduction in cerebellar weight, and a transient reduction in body weight after only a single day binge (Goodlett et al., 1989). This highlights the extent of damage that is occurring – enough to reduce the weight of entire brain structures such as the cerebellum – a crucial relay centre for many movement and cognitive functions.

This study used an intragastric intubation model to administer alcohol via 2 intubations to pups on PN6. Animals were euthanized on PN365 and brain tissue prepared for stereological analysis. A 49%, 38% and 26% reduction in CA1 pyramidal cell number relative to control was exhibited in E6, E5 and E4 animals respectively and all were significantly different to control animals. Further, the number of pyramidal cells present was loosely correlated with the peak BEC – indicating the higher the BEC, the more neuronal loss occurred. If an animal has lost half of the cells in the CA1 area of the hippocampus this must have severe implications for the intrinsic and extrinsic connections of that area within the brain, and for the functional behavioural output and affective responses that area is implicated in. As these neurons are post-mitotic, it may be of interest for future studies to elucidate why these CA1 and Acc neurons show such a high degree of susceptibility.

The reduction in neuronal number in the Acc as determined in this study was similar to the dose-dependent deficit seen in the hippocampal CA1 area, however not to the same extent. A 28%, 24% and 5% reduction in Acc neurons compared to IC animals in the E6, E5 and E4 groups respectively indicate this area of the brain is also vulnerable to the neurotoxic effects of ethanol during the third trimester. The 5% cell loss seen in the E4 animals was not significantly different from IC animals, however it is important to note that the lack of statistical significance seen here does not necessarily correlate to a lack of biological significance. Although a 5% loss seems minor, analysis did not assess loss within specific layers of the cortex, and if the loss is focussed over a particular layer within the Acc this may have implications for the neuronal circuits the Acc is connected with. In this way, even a small change in total cell number could contribute to subtle, yet important deficits in social and emotional processing, however
further studies are needed to investigate these putatively subtle changes as this was beyond the parameters of the current study. It is the children that have been exposed to moderate amounts of ethanol during pregnancy that often go unnoticed and hence do not receive the intervention needed to help them during childhood so problems do not persist into adulthood. Profound neuronal loss in the Acc may equate to a reduced ability to process and respond to subtle social cues, or inhibit or elicit variable responses depending on the situation. It is these aspects of social function that are more important than one might think, given the high level of interpersonal skills needed in the network driven society both children and adults now operate within.

It is important to note that there was only a significant neuronal deficit when the mean peak blood ethanol concentration was approximately 400mg/dl or higher. This can be equated to around 250mg/dl in the human, based on comparative LD50 ethanol doses in the rat and human – which would occur in a social binge drinker (Wiberg et al., 1971, Watson and McDonald, 1999b). If some mothers are drinking to this extent during the third trimester, this may pose a risk for a similar scenario of neuronal death in the developing foetus.

Children and adults who have been diagnosed with FASD show functional deficits in many of the domains initiated and regulated by the hippocampus and Acc, including learning and memory, social inhibition, and hyperactivity (Streissguth et al., 1994, Connor et al., 2000, Pei et al., 2008, Willoughby et al., 2008, Coles et al., 2011, Rasmussen et al., 2011). Therefore the neuronal loss seen in this study may be an underlying cause of some or all of these functional deficits in humans also. MRI studies of individuals with FASD have clearly shown reduced brain volumes in a number of regions including the hippocampus and prefrontal cortex (Coles et al., 2011). Thus future studies must ensure that the brains of FASD patients become available for post-mortem examination in a manner similar to the investigations in this thesis. It is only in this way that we will be able to align the findings from animal model studies to real world scenarios. Retrospective studies are important in the human FASD research field, however the reliability of questionnaires and variation in dosing makes this type of research inherently confounded – however in most situations when a diagnosis is under consideration this is the only available information. If the post-mortem studies can be combined with retrospective information regarding dosing, more robust conclusions can be made with regard to the functional impact of a structural neuronal deficit.
Such a profound cellular loss in these critical brain regions after just a single ethanol binge must be further verified in larger animal studies so that the deficits seen in humans can be more reliably attributed to neuronal death during development. Larger mammals such as monkeys and sheep are a feasible and reliable animal model in which this could be achieved, and if the same deficits are seen in these models they can be more easily extrapolated into humans (Cudd et al., 2007). This would facilitate the development of more effective education programmes to inform women on the scientific basis of the “alcohol and pregnancy” health warning, and be used when counselling to pregnant women is undertaken by health professionals. Current advice is often based on the FAS model in which the first trimester is deemed the most dangerous period. However it is becoming evident that both the second and third trimesters are periods of development not exempt from alcohol-induced damage, with the third trimester appearing to be a time of extreme vulnerability with the disadvantage that dysmorphic markers of alcohol exposure will not be evident. Scientific data that facilitates this is important as the functional deficits of developmental alcohol exposure are real and extremely important to recognise early.

8.2 Play behaviour and anxiety in rats exposed to a single ethanol binge on PN6

The hippocampal CA1 area and the Acc have a large number of reciprocal connections with brain areas such as the medial thalamic nuclei and the hypothalamus (van Groen and Wyss, 1990, Jay and Witter, 1991). As extensive cell loss in the CA1 and Acc is evident after a single binge, this entire network and functional output is also likely to be compromised. To date there have been no comprehensive investigations of the social and behavioural anomalies in rats exposed to a single ethanol binge during the third trimester. Both social skills deficits and related behavioural anomalies have been reported in children with prenatal alcohol exposure (Streissguth et al., 1991, Thomas et al., 1998c, Roebuck et al., 1999, Rasmussen et al., 2011) that can be inhibiting in children, leading to more serious problems later in life – while the underlying cause often goes undetected.

Thus the final part of this study used an animal model of social play fighting to investigate these important social interactions in a natural habitat. It was of importance to use a test that did not require the animals to endure forced responses or incentive
based tasks such as operant conditioning, as although a measure of frontal cortical function this is not a measure of social interaction. It was also vital to not have to use food deprivation as part of the paradigm as although this is always a component of the control group, it may act to confound alcohol treated rats but not controls. Play behaviour in rats is innately rewarding and has been shown to be the foundation for stable social relationships during adulthood (Panksepp, 1981). Animals were housed in quadrads, and video recorded for ten minutes in pairs during both adolescence (PN32-34) and adulthood (PN80-82). Following this, animals were tested on the elevated T maze (ETM).

The first important finding from this study was that all alcohol exposed groups showed a higher frequency of attack than SC animals during adolescence. This is in concordance with human data which show hyperactivity and impulsivity in alcohol-exposed children (Coles et al., 1997, Connor et al., 2000, O'Malley and Nanson, 2002a, Hellemans et al., 2005, Greenbaum et al., 2009). However, the IC animals also showed significantly higher levels of attack than SC animals. This suggests that either the intubation procedure has caused anomalies in the brain due to stress-induced trauma, or the IC animals have responded differently to this particular social experience. The dam may have neglected the IC animals to the same extent as the ethanol-exposed animals during development due to ultrasonic stress induced high-frequency vocalisations given off by the intubated animals, resulting in abnormal brain development in the postnatal brain growth spurt. If the intubation has resulted in an adverse stress response in the pup, the dam may not retrieve the animal and take it back to the nest. However even ethanol-exposed animals do not induce changes in maternal behaviour due to ultrasonic vocalizations, even though heightened levels of vocalization have been shown on PN5 following prenatal ethanol treatment (Marino et al., 2002). Although this maternal behaviour was not quantified following intubation, it may be an interesting aspect to analyse in future studies to try and elucidate the changes in behaviour seen here in intubation control animals. Recent findings from Washburn and colleagues (2013) found that an infusion of glucocorticoids to intubation control animals – used to mimic an increase in cortisol at the time of ethanol intubation – had no effect on Purkinje cell number suggesting that stress alone is unlikely to result in abnormal behaviour (Washburn et al., 2013). This intubation effect has not been documented in the literature, possibly indicating that this is the first study to reveal differences between IC and SC animals.
IC animals also displayed anomalies in behaviour similar to ethanol-effected animals on a number of other defensive parameters, suggesting this was not just a random experimental error. Interestingly, it was the E4 and IC animals that showed a higher probability of supine defence than SC animals when attacked during adolescence. As discussed in chapter 5, it is likely that the E4 and IC animals were displaying normal behaviour, and it was the SC animals that were behaving abnormally as the probability of SC animals going supine was well below the averages reported in the literature (Pellis and Pellis, 1990). This suggests the possibility that housing the SC and alcohol animals together has altered the normal development of SC animals. There were not sufficient animals in the study to determine if the effect on SC animals was greater in quadrads where pairing was with the ethanol-animals exposed to a higher dose but it is worthy of consideration.

A finding of perhaps the most importance in this study is that the significantly higher rate of attack in all alcohol treatment groups relative to SC animals was attenuated during maturity, indicating a level of functional recovery in ethanol-exposed animals. Circuits that were damaged following ethanol exposure may have undergone a compensatory change, and plastic changes may have occurred in other brain regions to allow recovery of behavioural function during maturity. Only two other studies to date have investigated the effect of neonatal alcohol exposure on play behaviour, and both studies used a chronic ethanol-exposure regime, and only analysed play behaviour during adolescence, not adulthood also like the current study (Meyer and Riley, 1986, Lawrence et al., 2008). Meyer and Riley (1986) found that male rats show a decrease in the typical frequency of attack, while females showed an increase in frequency – suggesting a sexually dimorphic effect of ethanol on play behaviour. This study was however very different to the current study in many aspects. For example, the play fighting analysis was recorded in real time with the experimenter sitting in the testing room, recording attacks and defences in real time. This increases the chance of scoring error quite significantly, as each bout happens extremely quickly and it is very difficult to differentiate each animal during a high-speed play fighting sequence. Also, animals were tested during the light cycle – which would decrease the rate of play, as rats are less mobile during the light phase due to their nocturne sleep/wake patterns. Therefore it is difficult to make direct comparisons with the current study due to ethanol exposure paradigm, analysis protocol and time of testing during the sleep/wake cycle. The study by Lawrence and colleagues (2008) also used a chronic three-trimester model of ethanol
exposure, and did not find any differences in the frequency of attack, rather an increase in the frequency of supine defence by ethanol-exposed animals – a finding that partially agrees with the results from adolescent data in the current study. However in the fore-mentioned study an attack was defined as any contact made with the ventral surface of one rat, including both the mouth and the paws, onto the entire dorsal surface of the play partner, except for the area around the tail. This is a very loose definition of a play fighting contact, normally contact on the nape of the neck, and thus makes it difficult to compare the data with that of the present study (Pellis and Pellis, 1987). The effects of ethanol on play behaviour clearly need to be investigated further, and following from the results of the current study and that of Lawrence and colleagues (2008), it may be prudent to specifically analyse supine defence and frequency of attack, and how anomalies can be ameliorated through behavioural and or pharmacological interventions. These social play findings are consistent with that of Stevens and colleagues (2011), who found children with FASD displayed abnormal affect processing and social perspective taking when compared to typically developing children. When combined, this may in part explain the lack of behavioural regulation exhibited in alcohol-exposed children, and if future studies can begin to elucidate the exact stressor, social situation or mechanism by which these differences are seen then more a more focused treatment approach can be formulated (Rasmussen et al., 2011, Stevens et al., 2011).

Ethanol-exposed animals showed an inability to acquire inhibitory avoidance on the ETM. Although this finding was not significant, it was apparent the ethanol-exposed animals did not understand the threat of the open arm, and successive trials failed to produce increasing latencies to exit the enclosed arm. The E6 group took significantly longer than both IC and SC groups to leave the enclosed arm in the baseline trial of inhibitory avoidance. Although not typically described as a finding of generalized anxiety in itself (Zangrossi and Graeff, 1997), this baseline trial anomaly poses interesting questions as to the cognitive and emotional state of the high dose animals – and a similar ratio between baseline and trial 1 has previously been employed in the literature to elucidate subtle differences (Trivedi and Coover, 2004). E5 and E4 groups also took noticeably longer than control groups to leave the enclosed arm on the baseline trial, although this was not significant. This indicates that the ethanol-exposed animals are more apprehensive about entering the open arm – an environment with no thigmotaxic properties, and hence a much more aversive experience. One-way escape
testing revealed that the E5 animals took significantly less time to escape from the open arm in trial 2 relative to baseline than both control groups. This indicates these animals may be displaying signs of panic disorder, another anxiety disorder characterized by recurring panic attacks – often initiated by open spaces. These findings are consistent with human data that show people diagnosed with FASD are prone to depression and anxiety – related disorders (Hellemans et al., 2010).

Taken together, these data indicate animals exposed to a single ethanol binge on PN6 perform surprisingly well on behavioural tasks involving social interaction and fear and anxiety related situations considering the substantial neuronal deficits that exist in key brain regions. These animals showed subtle anomalies in both play behaviour and anxiety levels, however the social deficits seemed to be resolved as the animals aged – an encouraging sign for FASD children with behavioural and social abnormalities. A developmental compensation must have occurred in the brain, as a permanent cell loss of the magnitude found to both the hippocampus and the anterior cingulate cortex surely must have an effect on function. Most of the previous work on social play behaviour has used experimental paradigms with a focal lesion in the brain, and therefore the effects seen in animals are easily attributed to the structural damage. However in the current study, cell loss was diffuse, and likely affected many brain structures – not only the ones specifically investigated. This makes it difficult to isolate certain behavioural anomalies, and also if diffuse damage has occurred in the brain – then perhaps everything is “downsized”, and still able to function relatively normally. It may be that alcohol – exposed children and adults are relatively comfortable in many social settings, until a stressor or novel event occurs and the brain is unable to compensate for the change in dynamic – and then the subsequent affect may be abnormal or detrimental to the situation.

The relatively mild effects on behaviour as seen in this study after a single ethanol binge indicates that if a mother has a binge drinking session during the third trimester of pregnancy, the subsequent damage to the foetus may only be subtle and possibly repairable with diagnosis and intervention. However it is essential that the mother cease drinking immediately to avoid more serious damage.
8.3 Other considerations

As the gestational period of the rat is 20 days, and the equivalent human gestational period is 280 days, then this may have implications with regard to the length of ethanol exposure being of increased duration in the rat relative to the human if the one-day model is used. The animals used in these studies were essentially intoxicated for a portion of their gestational period that, if converted to the human period would be a lot more than one day. This is one issue that is difficult to overcome, and although the animals are exposed for a proportionately longer period of gestation, the blood ethanol levels obtained are similar to that occurring in a social binge situation in humans. However this is a very modest exposure duration compared to many animal models that still purport to be modelling human drinking during pregnancy (Goodlett and Johnson, 1997, Klintsova et al., 2007).

As the third trimester equivalent in the rat is from PN day 1-10, this also means that the route of ethanol administration must be direct to the new-born rat (via intragastric intubation, or through delivery to the dam and secondary delivery through the milk). This poses a slight difference in the way the animal is processing and metabolizing the ethanol – as in the human it would be via cross-placental route, and thus not undergoing first pass metabolism in the portal circulation. For this reason, as stated earlier – it is important for these studies to be replicated in mammals where a greater control and comparability over ethanol delivery and gestation can be attained.

Further research into the social and behavioural ramifications of a single ethanol binge in the third trimester is essential to determine the exact extent to which the neuronal loss seen during development has impacted on function. A paucity of literature in this area reflects a relative neglect of an on-going problem within New Zealand and other countries, and despite large advertising and awareness campaigns, mothers are continuing to drink to dangerous levels during pregnancy. As a result, children that may not have received a formal diagnosis of FAS or FASD are not being fully evaluated and tested for learning and social disabilities that may have dire consequences throughout their lives. Screening and intensive awareness programs for at-risk mothers are essential to begin to address an entirely preventable cascade of events, and for this to work efficiently we need government input and funding and collaboration between clinicians and scientists.
8.4 Conclusion

Drinking during pregnancy remains an on-going social, economic, political and medical issue for New Zealand and many other countries. Many of the effects of drinking early in pregnancy during the first trimester have been thoroughly documented. However there is a belief that after this period the risk diminishes and anomalies are not seen in the offspring. This thesis has contributed a piece of evidence to the much-needed pool of literature in this area of child, adolescent and adult development after a prenatal insult during the third trimester. The findings were in part what were hypothesized, such as the massive neuronal loss after a single ethanol binge on PN6 – however other findings such as the social play abnormalities in both ethanol-exposed and control animals clearly warrant further investigations. Novel behavioural testing regimes, intensive structural brain analysis and the introduction of mammalian models are needed to bridge the gap between human and rodent – and maybe then that mother will decide to wait three more weeks before she takes that glass of wine.
Chapter 9. References


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Chapter 10. Appendix

10.1 – Histological protocols

Cryoprotectant:

150ml Ethylene glycol
150ml Gycerol
100 ml 0.3M Phosphate buffer (pH=7.2)
100ml milliQ water

Ethylene glycol:

150ml Gycerol
100 ml 0.3M Phosphate buffer (pH=7.2) - solution made up in lab
100ml milliQ water - ddH2O

0.3M PHOSPHATE BUFFER to make up one liter, pH = 7.2
NaH2PO42H2O - 46.8g
if using monohydrate - 41.39g
Na2HPO42H2O - 53.4g
if using anhydrous - 42.59g

Make up to 900ml, it is a very acidic solution so you will need to use NaOH to bring pH up. It takes around 6g of NaOH pellets. I used in excess of 25 pellets, were a little wet.

Cut sections and put a number in each well in a 24/96 well tissue culture plate, cover with parafilm and the lid and then store it in a -20C freezer.

Sections retain antigenicity for NeuN, c-fos and zif for at least 9 months.

4% Paraformaldehyde solution:

4% Paraformaldehyde in 0.1M Phosphate buffer

4 g paraformaldehyde (do not breath fumes)
add to 50 ml distilled water
Heat to 60°C - dissolve, add 1-2 drops of 1M NaOH to clear the solution
Cool - add 50 ml 0.2M phosphate buffer.
10.2 – Immunohistochemistry protocol

Free floating immuno protocol

Use with NeuN and with GFAP
All steps - at room temperature unless stated otherwise
Sections washed in phosphate buffered saline between each step.
All processing to be done in wells, withdraw solutions carefully with pipette.
All processing to be done on a slow moving shaker table.

Day 1
• Rinse 2 x 10mins 1xPBS
• Remove aldehyde residue by quenching in 0.1M glycine for 20 mins
• Rinse 2 x 20min 1xPBS
• Block (100µL HIGS in 900µL 1% PBS/BSA) for 1 hour
• Remove block from sections
• Apply monoclonal NeuN primary antibody (diluted 1:200 in 1% PBS/BSA) for 48 hours @ 4°C

Day 3
• After primary incubation, remove primary into antibody waste container
• Wash 3 x 1 hour with 1xPBS
• Block (100µL HIGS in 900µL PBT for 1 hour)
• Remove block from the sections
• Incubate in secondary: biotinylated goat anti-mouse 1:200 diluted with 1%PBS/BSA(PH7.4), incubate 3 hour at RT. Remove secondary antibody to waste container
• Wash 1 hour with 1xPBS
• Wash 1 hour with 4xPBS
• Wash 1 hour with 1xPBS
• Wash in 0.5% H₂O₂ in Methanol (3.33 ml 30% H₂O₂ (or 2 ml 50% H₂O₂) adding in 200 ml methanol) for 15 min at room temperature (0.83 ml 30% H₂O₂ in 50 ml methanol).
• Rinse in 1xPBS 10 min to clear out methanol.
• Rinse in 1XPBS 30 min x 2 to clear out methanol
• Incubate in biotinylated Strepdavidin-HRP diluted 1:100 in 1xPBS for 1 hour at RT (on fridge 4th floor lab)
• Rinse in 1xPBS 30 min x 3.
Visualisation:

- 1: Visualize with DAB in diluted peroxide buffer (1: 10), 10 min at RT (1 min is OK).

2: **Nova Red stain**
   - Add 100µl to each section and incubate for exactly 15 minutes
   - Put Nova Red waste in labelled Waste container

The staining solution contains a soluble substrate and hydrogen peroxide, which will be converted by the peroxidase into a red precipitate. The dye precipitates around the secondary antibody.

The length of this step is determined empirically and is influenced by the concentration of the primary antibody and the abundance of the antigen. Too short an incubation will fail to reveal antigenic sites. Too long, will lead to false background stain.

**Milli-Q Water**

* 3x 1 mins in "H₂O" Stops the reaction.

Sections were then mounted onto gel-coated slides

**Options to mount sections**

*Dehydration and mounting in Xylene*

  - Harris Haematoxylin: 3 dip is enough
  - Wash in water: 3 min
  - Acid alcohol 6 sec
  - Wash in water: 3 min
  - Blue: 6 sec
  - Wash in water: 3 min
  - 75% Ethanol: 6 sec
  - 95% Ethanol: 8 sec
  - 100% Ethanol: 8 sec
  - Xylene: 1 min x 2

Sections then cover slipped with DPX before visualisation under light microscope (Olympus AX70).

**OR - Coverslip**

* place one 1 drop Glycerol/PBS (little green topped vial)
* Fold in filter paper circle to blot excess
* Seal edges of coverslip with nail polish

- biotinylated goat anti-mouse 1:200 diluted with 0.1 M PBS (PH7.4), incubate 3 hour at RT (5 ul aliquots, 9B LFB –80 ºC freezer)
- monoclonal NeuN primary antibody
- biotinylated Strepavidin-HRP - freezer of LFB –80 ºC 15 D & E,
(for 1:100 dilution 10 ul aliquot make up 1 ml, 20 ul aliquot make up 2 ml).

**Solutions:**

**Stock:**

1% Thionin:

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<th>Thionin</th>
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<td>ddH2O</td>
<td>100ml</td>
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Mix well on stirrer

0.1 M Acetate Buffer pH 3.5:

| 0.2M Sodium Acetate | 100ml |
| ddH2O               | 86ml  |
| Glacial Acetic Acid | Approx 13ml |

Add GaA until pH 3.5, then make up to 200ml with ddH2O

**Solution:**

| 1% Thionin       | 25ml  |
| Acetate Buffer pH 3.5 | 25ml  |

Mix, Filter and use

Solution will stand for repetitive use for several weeks, re-filter before use.

**Thionin – Staining**

1. **Staining**

<table>
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<td>Solution</td>
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2. **Differentiation**

| Tab water | 2 min |
| Alcohol grading (50, 75, 95%) | 30 secs each |
| 100% Acidified Alcohol | 10-15 min |
| 3dr. GaA/50ml or 6ml/500ml | |
| 10dr. GaA = 2ml | |
| 100 % Alcohol | 2 min |
3. Mounting

Cover section with DPX
Allow coverslip to settle down slowly

Let slides dry (in fume hood) at room temperature for about half an hour before putting them into the incubator at 37°C.
10.3 – Data recording tables

**General one-day intubation:**

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Treatment period

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185
10.4 – Intubation protocol

EtOH Intubation Protocol for Rat Pups

<table>
<thead>
<tr>
<th>Items Needed for Intubation Procedure</th>
<th>Items for Paw-Coding Pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>• 95% EtOH (for sterilization purposes)</td>
<td>• 1-30g x 3/8” needle (or 26g)</td>
</tr>
<tr>
<td>• 1-Weigh scale</td>
<td>• 1-1cc syringe</td>
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<td>• 2-3 Pup compartments/containers and warm cloth</td>
<td>• India ink</td>
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<tr>
<td>• PE-10 and PE-50 polyethylene tubing (Clay Adams Brand) that has been made</td>
<td>• latex gloves</td>
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<tr>
<td>into intubation cannulae (best to have a different one for each dose if</td>
<td>• 95% EtOH [for rinsing ink]</td>
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<td>doing different doses in one litter.)</td>
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<td>• 1-22g x ½” needle (for making cannula)</td>
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<td>• 1-Heating pad</td>
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<td>• 1-1cc slip-tip syringe</td>
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<tr>
<td>• 1-syringe needle to get alcohol milk solution out of bottle</td>
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<td>• Corn oil</td>
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<td>• Intubation Volume Look-up Sheet</td>
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<td>• Milk Solutions</td>
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<td>• Tap or sterile water</td>
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<td>• 70% Isopropyl alcohol in container</td>
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<td>• Kim wipes and/or paper towels</td>
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<td>• permanent markers of different colours to mark pup tails</td>
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<tr>
<td>• 1-30g x 3/8” needle (or 26g)</td>
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</table>

I. INTUBATION TUBE PREPARATION (see Figure 1 below)

A. Use a pair of fine iris scissors to cut a 20-30cm long piece of PE-10 tubing. Snip off one end at an angle; this will be used as the insertion end to the PE-50 tubing piece.

B. Cut a 10cm piece of PE-50 tubing and attach this to the insertion end of the PE-10 tube until it is snug.

C. Attach the other end of the PE-50 tube to a 22g needle. Do not puncture the tubing, it helps to slightly blunt the sharp edges of the needle by rubbing on sandstone before inserting needle into PE-50 tubing. Check that the tubing does not leak at the joins.

D. Snip off the intubation end so that it is “flat”; this may help prevent causing internal bodily damage to the pups. Flare the end very gently in flame.

![Figure 1: Diagram of intubation setup](image)
Table 1: Typical Feeding Schedule for Postnatal Day (PD) 4–9 Exposure Studies (X = Event Occurs)

<table>
<thead>
<tr>
<th>PD</th>
<th>Paw Mark/Assign Pups</th>
<th>Sess 1 EtOH/SHAM</th>
<th>Sess 2 EtOH/SHAM</th>
<th>Sess 3 Collect Blood</th>
<th>Sess 3 Milk only/SHAM</th>
<th>Sess 4 Milk only/SHAM</th>
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<tbody>
<tr>
<td>4</td>
<td>Check pups and sex</td>
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<td>X</td>
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<td>7</td>
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<td>milk</td>
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<td>8</td>
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<td>X</td>
<td>X</td>
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<td>X</td>
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<tr>
<td>9</td>
<td>Paw mark</td>
<td>milk</td>
<td>milk</td>
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II. INTUBATION PROCEDURE

A. Feeding Schedule

The pups are given 2 feedings per day of ethanol-milk solution at the appropriate dose, based on body weight, see sheet. (The 6g/kg and 5/25g/kg ethanol animals are then given additional 2 milk only feedings on the day of alcohol exposure and maybe one of two the next day depending on body weight and if milk in stomach). To maintain consistency in feeding schedule, the first intubation should occur in the early morning (e.g., 9 AM) and subsequent feedings every two (2) hours (see Table 1). It is ideal to give the first extra feed at time of blood collection for BEC but sometimes stomach is still too full. Come back and do feeding later. Have been giving additional feeds at 4 hours post 2nd alcohol feed and then at 4 to 5 hours after that. It is okay to do these feedings at roughly these times to fit in with your life. This regime needs to be used for all animals, chronic studies, e.g. behaviour and also for acute cell death studies.

After each use, refrigerate the milk.

- To maintain the freshness of the milk substitute solution it can be aliquotted to 10 ml serum bottles with the appropriate volume of 95% ETOH (e.g., for 5.25 g/kg dose in 10 ml total milk, add 1.43 ml 95% ETOH). This also prevents any contamination getting into the main bottle from repeated use.

We attempt to make up enough of each ethanol solution so that any pups that will be compared in different cohorts will receive the same solution.

NOTE. The milk solution is intralipid intravenous food replacement.

B. Procedures

1. Remove the milk solution from the refrigerator and allow to sit in warm water. Don’t overheat!
2. On PD 4, remove all the pups from the mother and place them in a container that is situated on a heating pad [low setting]. For each pup determine their sex (i.e., **use anogenital distance as a marker**: males = longer distance than females). Try and get 6 of each sex but do not need to weigh or assign to groups until the morning of treatment. If extra pups in one litter and short in another can cross foster but mark animals you foster on and record litter they are from. Remove all pups from dam you are going to foster pups to, rub some feces on the pups to be fostered and place all pups back to mum together. Generally this works.

On the day of treatment assign pups to the appropriate treatment group. Try to match pups in alcohol, IC and SC groups on weight and across sex. Do not always have large animals in highest alcohol group but spread groups across differences in body weight. Record this information in the data sheets, and keep in a very safe place, a folder. Make a copy of all data sheets when complete and give the copy to R Napper.

3. Use a permanent marker to give them temporary ID’s (e.g., #1, 2, 3, etc.) as 1, 2 or 3 lines on the top of the tail using different coloured pens, red, green and blue as black and blue can look the same when getting worn. . **See Section IV for paw-marking details.**

4. Weigh each pup and record the information in the logbook. Always double-check the animal #.

5. During the first intubation session, determine the appropriate EtOH milk intubation volume for EtOH-designated pups. Use the pup intubation volume sheet. If no sheet is available,

then multiply the pup’s body weight (g) by 0.0278 and round off to the nearest decimal. The same intubation volume is used for all intubation sessions within the day, so body weights need only be taken once daily prior to intubation.

6. If the intubation tubing has been sitting in isopropyl alcohol, then load a 1cc syringe with tap or sterile water and flush it out. Check for any micro-leaks at this time.

7. If the EtOH milk solution was prepared in a serum bottle that has been stoppered and sealed, then use a 26g needle attached to the 1cc syringe to withdraw the appropriate volume of EtOH milk solution (or milk only). Draw out a little more milk than normal and tap out any air bubbles from the syringe.

8. Attach the syringe to a 22g needle bearing the intubation tube. Tap the syringe again to remove air bubbles from the needle reservoir. Flush out enough milk until it reaches the appropriate intubation volume indicated on the syringe.

9. Prior to intubating a pup, visually determine the appropriate tube insertion distance (the tip of the PE-
10 tube should stop short of reaching the stomach. Mark the tube with a permanent marker at the gauged distance.

10. Dip the PE-10 tube into corn oil and begin inserting the tube.

**Some pointers (it’s a feel thing!)**

- Hold tubing so its natural curvature follows the curvature of the pup.

- Use your thumb and middle finger to hold the pup by each side of its temples. Use your index finger to stabilize its head.

- Fingers should be holding the tube near the tip to maximize control.

- Insert tubing into pup's mouth slightly at the midline and proceed towards its throat.

- Do not jab the tube into the animal’s throat, but rather place slight pressure at the entry into the esophagus and wait for the animal to open its mouth further in response to the stimulation. When it has opened its mouth further, then that becomes the most opportune time to place even more pressure on the tip of the tube to move forward.

- As the tubing goes down the esophagus and into stomach, there should be no resistance. If resistance is met, remove tubing and start over.

- Stabilize the pup by holding it firmly – such that its paws are not moving in any way but avoid causing it discomfort. When the tube is inserted properly in the pup and it is stabilized, depress the plunger **slowly** until the milk solution empties. There should be a normal parasympathetic response to the milk. The pup should relax and stop/slow down any squirming.

- Upon completion, **slowly** remove the intubation tube from the pup and return it immediately to its littermates. Efficient intubations can be completed in 30 sec or less per pup.

**EXTRA NOTES:**

- Always keep your eyes on the pup while intubating to correct any displacement of the original tube stop distance and/or improper solution flow through the intubation tube. There should be very little flow resistance while depressing the plunger.
• There may be cases where the pup has been damaged by the intubation process—either by intubating some amount of solution in the lungs or by stabbing through the esophageal wall, through the cardiac sphincter, or other internal organs/tissue. In these cases, the pup has a VERY low chance for survival and should be euthanized.

11. On the first EtOH pup for each litter, note the time it was intubated in the logbook and use this time to determine the beginning of the next intubation period. It is recommended that you intubate the pups in chronological order, particularly on days when blood samples are taken.

12. For sham intubated control pups, they receive the intubation procedure only without milk or ETOH milk. The tube should remain in them for several seconds (about the same duration that it takes to intubate an EtOH pup).

NOTE: Removal of pups from dams for any prolonged period of time may cause unwarranted stressor effects, therefore, the intubation procedure should be done as swiftly as possible so as to return the pups [as a litter] back to the mother. Do not leave any pup isolated from its littermates for long either—especially if you happen to be distracted for some reason. Note that the pups that have been assigned to the suckle control group get removed from the dam with the rest of the litter but the only manipulation they receive is weighing daily.

13. Flush all syringes and tubes with water between intubations and store them in 70% isopropyl alcohol. The isopropyl alcohol should also be flushed out with water prior to each use. Use a new syringe daily. To save preparation time, intubation tubes can be stored and reused for several days (or even litters) – use your BEST judgment in determining what are good and bad tubes.

14. Refrigerate the milk solution(s) immediately between uses but warm prior to use.

15. Clean up your area in between sessions (e.g., use 95% EtOH on countertops and weigh scale, wipe away urine, and remove feces, etc.) and keep materials confined to a space. The SL surgery room is shared space, so be considerate of others!