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The effects of ethanol on memory and neuroplasticity in a vertebrate and an invertebrate model of learning

Ian Sloss

Submitted for the degree of Doctor of Philosophy

University of Sussex

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Declaration

The work in this thesis is entirely my own, except where references to the work of others are acknowledged. This thesis has not been and will not be submitted in whole or in part to this or any other university as part of a degree.

Signature:

[Signature]

Ian Sloss
Acknowledgements

I would like to thank my supervisors, George Kemenes, Tamzin Ripley, Pete Clifton and Dai Stephens for their support and guidance throughout my time. I would like to thank Sandra and Dave for their support with the mice, and Mikey and Souvik for their support with *Lymnaea*. I am also very grateful to the Shaylers for their selfless support during my final year, and to my friends and family for keeping me full and dry.
Abstract

Binge drinking is characterised by cycles of ethanol intoxication and withdrawal and is thought to be highly deleterious for the normal functioning of the nervous system. The behavioural and neurophysiological consequences of rapid escalation of blood alcohol concentration and subsequent withdrawal, and their effects on learning and memory and underlying neural circuitry can be studied in suitable animal models. Here, spatial and instrumental learning as well as hippocampal LTP were assessed in C57BL/6J mice for the effects of adolescent intermittent ethanol (AIE) and other ethanol treatments. AIE treatment did not impair spatial or non-spatial memory when tested in adulthood. However, if mice were trained whilst intoxicated during AIE treatment, spatial memory was impaired. Post-training injections of ethanol impaired performance in operant conditioning. A rapid rise and fall in ethanol concentration, prior to stimulation, blocked LTP induction in drug naïve hippocampal slices; an effect that was not seen if the ethanol concentration was gradually increased and decreased. Moreover, AIE treatment caused an NMDA receptor-dependent transient increase in hippocampal LTP. The second part of this study used a novel molluscan model Lymnaea stagnalis and demonstrated that high concentrations of ethanol blocked acquisition and retrieval of an associative memory. However, if acquisition occurred in the presence of ethanol then memory could also be retrieved under ethanol, demonstrating ethanol state dependency. By utilising the cerebral giant cells, a modulatory neuron type with known involvement in memory formation, it was found that ethanol reduced the tonic firing frequency as well as the peak-to-trough and half-width parameters of individual action potentials. The development of in vivo and in vitro ethanol treatment and test protocols, and the findings based on their use, open up new avenues for future systematic investigations on ethanol’s effects on behaviour and underlying neural circuitry in both vertebrate and invertebrate model systems.
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## Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>APV</td>
<td>2-amino-5 phosphonovalerate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BEC</td>
<td>Blood ethanol concentrations</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu ammonis area 1</td>
</tr>
<tr>
<td>CA2</td>
<td>Cornu ammonis area 2</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu ammonis area 3</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium calmodulin kinase 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CED</td>
<td>Cambridge electrical design</td>
</tr>
<tr>
<td>CGC</td>
<td>Cerebral giant cells</td>
</tr>
<tr>
<td>CIE</td>
<td>Chronic intermittent ethanol</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CR</td>
<td>Conditioned response</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>CTA</td>
<td>Conditioned taste aversion</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory post-synaptic potential</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>fEPSP</td>
<td>Field excitatory post-synaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>c-aminobutyric acid-A receptors</td>
</tr>
<tr>
<td>HFS</td>
<td>High frequency stimulation</td>
</tr>
<tr>
<td>I/O</td>
<td>Input, output (referring to a curve)</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ITI</td>
<td>Inter trial interval</td>
</tr>
<tr>
<td>ITM</td>
<td>Intermediate-term memory</td>
</tr>
<tr>
<td>LTM</td>
<td>Long-term memory</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MK-801</td>
<td>(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate</td>
</tr>
<tr>
<td>mPFC</td>
<td>Medial pre-frontal cortex</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NS</td>
<td>Neutral stimulus</td>
</tr>
<tr>
<td>OR</td>
<td>Object recognition</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PND</td>
<td>Post-natal days</td>
</tr>
<tr>
<td>PSD</td>
<td>Post-synaptic density</td>
</tr>
<tr>
<td>PTP</td>
<td>Post-tetanic potentiation</td>
</tr>
<tr>
<td>PTX</td>
<td>Picrotoxin</td>
</tr>
<tr>
<td>RPed1</td>
<td>Right pedal dorsal 1</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical package for social sciences</td>
</tr>
<tr>
<td>STM</td>
<td>Short-term memory</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage gated calcium channels</td>
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</table>
Chapter 1 – General Introduction

Alcohol use and in particular binge drinking is increasingly commonplace in society, particularly during adolescence and young adulthood (Miller et al., 2007). In the UK, the average weekly consumption of alcohol is greatest between the ages of 16 - 24, with males consuming 17.5 units and females 11 units of alcohol per week (Statistics, 2013). Binge drinking has a negative socio-economic impact through a range of effects including; alcohol-related deaths (Courtney and Polich, 2009), general accidents, physical and mental health problems, poor academic performance, increase in sick days, anti-social behaviour and violence (British Medical Association, 2009, Healey et al., 2014). Certain brain regions like the temporal and frontal cortices are still under development in adolescence (Fein et al., 2013, Giedd et al., 1999) and could be at risk of damage or alteration due to repetitive bouts binge drinking (Spear, 2013, Squeglia et al., 2011).

Studying the underlying neurological effects of drinking patterns in adolescent humans is difficult due to a number of reasons, including; poor accuracy in recalling the duration and quantity of alcohol consumption (Sobell and Sobell, 1995, Leeman et al., 2010) and no control of the uptake kinetics of alcohol into the blood (Zimmermann et al., 2008). To explore the neurobiological effects binge drinking may have during adolescence it is useful to use animal models. However, since binge drinking itself is a human phenomenon, it is useful to model for its most significant feature, the rapid escalation of blood-alcohol concentration during a short time-frame, followed by a period of abstinence (Townshend and Duka, 2005, Stephens and Duka, 2008, Spear, 2015).

This chapter discusses the history and current thoughts of learning, memory and neuronal plasticity. As well as introducing the numerous actions that acute ethanol and repeated treatments of ethanol that aim to model for human binge drinking, has on nervous tissue. Special attention is made to highlight the differential effects that these ethanol can have between adolescents and adults. A review of the current vertebrate and invertebrate animal models used to investigate ethanol use is included. Also the aims and hypotheses of the thesis are defined.
1.1 Principles of learning and memory

Here, I will provide an overview of the most important principles and concepts of learning and memory in general. This initial overview will be based on human learning and memory but many of the same principles also apply to other animals, including many invertebrate species, which are therefore often used as models to elucidate the most fundamental and highly conserved mechanisms of learning and memory.

The ways in which we react to our environment is of the utmost importance to our quality of lives and even survival. Having cognitive faculties such as learning and memory allows us to shape our behaviour and adapt better to our environment. While learning allows us to acquire new knowledge, faces, places and survival skills, memory allows us to remember and act on, this acquired knowledge. Indeed, most of what shapes us as individuals is our unique set of experiences and their resultant memories. We learn that certain experiences are enjoyable and others must be avoided. Learning and memory are therefore at the centre of what makes us. However what memory actually is, is still being debated.

One of the earliest theories of learning and memory was put forward by 19th century philosopher Alexander Bain, who proposed that “for every act of memory, every exercise of bodily aptitude, every habit, recollection, train of ideas, there is a specific grouping or co-ordination of sensations and movements by virtue of specific growths in the cell-junctions” (Bain, 1855). Towards the end of the 19th century the eminent histologist and founding father of neuroscience, Santiago Ramon y Cajal produced famous images of neurons from most regions of the brain. He was a strong defender of the ‘neuron doctrine’, and he believed that the brain consisted of many nerve cells (neurons) that could communicate together by linked points (Ramon y Cajal, 1933).

In the early 20th century there were two conflicting theories of the biology of memory. One theory was the “cellular connectionist approach”, which was based on Ramon y Cajal’s ‘neuron doctrine’ theory. It states that as we learn the synaptic connections between our neurons become stronger and more efficient (Cajal, 1894). This later became renamed synaptic plasticity by Konorski, and will be covered in detail later. Another theory was the “aggregate field approach” supported by Karl Lashley and Ross
Adey (Kandel and Spencer, 1968). They suggested that memory is stored in the bio-electrical field that is generated by the close proximity of multiple neurons.

One of the first pieces of experimental evidence that attempted to answer whether memory was localised to certain brain regions or not, was by Wilder Penfield – a student of the famous neurosurgeon and neuroscientist, Charles Sherrington. Sherrington was famous for having had mapped the motor cortex of monkeys by stimulating brain areas and waiting to see a response from the body (Sherrington, 1906). Penfield continued Sherrington’s work by applying it to humans who were to undergo epilepsy surgery. By applying electrical stimulation to various locations on the surface of the cortex of anaesthetized but awake patients, Penfield was able to ask patients what sensations they felt upon each stimulation. Penfield found what he termed an “experimental response”, where patients would recall a previous experience after particular areas of their temporal lobes were stimulated. Although not conclusive, this was an indication that memory may be in fact localised and that the temporal lobe may be the site where memories are stored.

Memory can be thought of as having two major forms: implicit and explicit (see Fig 1.1 for diagram). Implicit (otherwise known as procedural or non-declarative) are physical or motor memories – things we cannot codify linguistically, such as how to ride a bike, learning to draw whilst looking in a mirror or a simple conditioned reflex reaction. Often these memories require some training or practise and involve building upon existing reflexes. Implicit memory can be further sub divided into priming, procedural, classical and operant conditioning, habituation and sensitisation (Sweatt, 2010, Kandel, 2000).

Explicit memory is a list of facts that we can recall such as our phone number or where we went to school as a child. Explicit memory can be divided into semantic memory which concerns all the facts we possess in our knowledge and episodic memory which is used to recall the events that have happened in our lives.
Figure 1.1. Hierarchical display of memory types. (Top) Subdivisions of human memory with their associated brain regions (Milner et al., 1998). (Bottom) Diagram of the interactions that subdivisions of memory have (Sweatt, 2010).
Another way memory can be characterised is into different temporal phases. Memory can exist as short-term memory (STM), intermediate-term memory (ITM) and long-term memory (LTM). STM lasts for only a few minutes after acquisition unless it is not converted into a longer form of memory and it is not dependent on transcription or translation. ITM is used to describe a memory trace that lasts for a few hours after acquisition and it is only dependent on translation but not transcription. LTM can last for days or even years and it is dependent on both transcription and translation (Sweatt, 2010).

1.1.1 Spatial memory

One of the most-studied forms of explicit memory is spatial memory. This type of memory is involved in locating oneself in one’s environment and recalling spatial maps (O’Keefe and Dostrovsky, 1971, O’Keefe, 2007). For example, an intact spatial memory is necessary for mice to successfully complete the Morris water maze, a water maze in which the subject has to learn the position of a submerged hidden platform relative to its surroundings, locating the platform allows the subject to stop swimming and remain above the water (Morris, 1984). Initially, the mice have no knowledge of the platform, but after sufficient training trials they learn the location of the platform relative to the extra-maze surroundings. This updates a spatial map of the maze and becomes consolidated in the mouse’s memory. In later trials the mouse can accurately locate the platform by using the surrounding environmental cues (Vorhees and Williams, 2014).

Various experiments have been conducted in order to locate the part of the brain involved with processing spatial learning and memory. In the 1970’s, John O’Keefe et al, recorded neurons in the hippocampi of freely behaving animals. This provided the first conclusive evidence for hippocampal involvement in processing spatial information. It was found that particular spatial locations or places activated certain neurons within the hippocampus, this phenomenon gave these neurons the name ‘place cells’ (O’Keefe, 1971). O’Keefe continued by describing hippocampal involvement in a neuro-cognitive map consisting of two systems: a place system, and misplace system. The place system contains cells that locate an individual’s spatial position in an environment. The misplace system contains cells that signal for a mismatch between the current perception and the stored memory of an environment. Mismatches trigger exploration in the subject and allow for an update in the place system (O’Keefe 1975). These days we know place cells
as hippocampal pyramidal neurons, and we know they are instrumental in spatial learning and memory (Vorhees and Williams, 2014, O'Keefe, 2007).

One of the most important early studies in memory research was that of patient H.M. Brenda Milner and William Scoville, both colleagues of the aforementioned Penfield, worked with patient H.M., who had suffered from severe epilepsy from a young age, and in 1957, at the age of 28, underwent surgery to remove a large portion of his temporal lobe, including the hippocampus, parahippocampal gyrus and the amygdala (Annese et al., 2014). The surgery was a success, insofar as he no longer suffered from such extreme seizures. However, H.M. could no longer form new long-term memories, and now suffered from anterograde amnesia (Annese et al., 2014). His knowledge of his childhood and other previously learnt memories remained intact, as did his ability to perform STM-dependent tasks. Moreover he was able to adequately perform implicit learning tasks and improve at motor procedures, but he could not remember having done the task before. From this case-study the hippocampus became a real target of interest in psychology and neuroscience who were interested in understanding memory. A more recent, non-invasive study of the human hippocampus was performed in London taxi drivers. London taxi drivers have to learn the layout of around 25,000 streets in the city of London, including additional places of interest (Maguire et al., 2006). In these studies they found that the right posterior hippocampus is activated when successfully recalling complex routes around the city (Maguire et al., 1997). Interestingly this region of the hippocampus also undergoes significant growth as a product of this training, resulting in the right posterior hippocampus to be a greater volume in taxi drivers than non-taxi drivers (Maguire et al., 2000, Maguire et al., 2003). This suggest that to increase the spatial memory of humans the brain adapts and actually increases the volume of the hippocampus in order to accommodate for necessary improvements in function.

Cases like H.M. and others like him, have contributed a great deal to the understanding of human memory (Annese et al., 2014). However, correlation and imaging studies of London taxi drivers do not provide the depth of knowledge that surgery patients have had. Nor as these surgery studies ethical to perform, or easily replicated in humans. Therefore, investigating the underlying neural, cellular and molecular mechanisms of memory is no easy task. To be able to truly understand how memory works in complex systems such as the human brain there first needs to be an understanding of the biology of memory in its simplest forms. Therefore a reductionist approach, investigating memory using ‘simpler’ mammals and invertebrates is needed.
1.1.2 Classical and operant conditioning

In the late 20th century this approach of conditioning a simple reflex was used in many simple model systems. These experiments were relatively simple and easily repeatable, and for the first time, it was possible to investigate the mechanisms that underlie memory. Many of these studies sought most of their inspiration directly or indirectly from Ivan Pavlov’s work on classical conditioning or Edgar Thorndike or B.F. Skinner’s work on operant conditioning. Therefore I will briefly describe those historical findings before going on to experiments of conditioning reflexes.

One of the pioneers of behavioural neuroscience and memory research in the late 19th/early 20th century was Ivan Pavlov, who is well known for his work on associative classical conditioning. He knew that dogs would salivate upon being presented with food. He called this an unconditional response (UR) to an unconditioned stimulus (US). He experimented with presenting various stimuli before the food was presented. He would ring a bell immediately before presenting the dog with food. The dog learned to associate the bell with the food (Kandel, 2000). Later Pavlov noticed that the dog no longer needed the food to start salivating, the ringing of the bell alone would cause the dog to salivate. In this example the bell has become a conditioned stimulus and elicits an unconditional response: salivation. This approach showed that a simple reflex could be conditioned (Kandel, 2000).

At the start of the 20th century after the pioneering work in learning theories from Pavlov and others in classical conditioning, Edgar Thorndike pioneered Operant conditioning with his postulated theory ‘the law of effect’. Thorndike experimented on cats. A cat was placed inside a puzzle box and a piece of fish was left outside the box to tempt the cat to leave. The cat would perform certain random acts and eventually find a lever to exit the box and reach the fish. This procedure was repeated and after a number of trials the cat’s duration inside the puzzle box would reduce, as the cat had learned the response of pressing the lever (Sweatt, 2010, Kandel, 2000).

“Of several responses made to the same situation, those which are accompanied or closely followed by satisfaction to the animal…will, other things being equal, be more firmly connected with the situation…; those which are accompanied or closely followed by discomfort…will have their connections with the situation weakened…The greater the
satisfaction or discomfort, the greater the strengthening or weakening of the bond." (Thorndike, 1898).

Arguably the one of strongest influences on operant conditioning was B.F. Skinner. One of the elements Skinner introduced to operant conditioning was that of reinforcement. Behaviours that were reinforced were continued, whereas behaviours that were not reinforced tended to not be repeated. For example, Skinner placed hungry rats inside a Skinner box. Randomly the rat would press a lever, and consequently be presented with a piece of food. After repeated pairings the rat learns to press the lever for food (Skinner, 1938).

Of the many studies that used these conditioning paradigms one of the best studied examples of conditioning a simple reflex was in the mollusc *Aplysia* (closely related to the invertebrate model we will use in this thesis, *Lymnaea*). The *Aplysia*’s gill withdrawal reflex consists of a sensory neuron to interneuron to motor neuron pathway. The natural reflex reaction in this animal is to protectively withdraw the gill when the animal is in contact with a potentially harmful stimulus (e.g. a predator touching its tail). But this behavioural response can be habituated by repeatedly stimulating the sensory neurons in the tail without harm occurring. With repeated stimulation, the response to the stimulation in the sensory and interneurons weakens and subsequently reduces the chance of stimulating a withdrawal reflex (Carew et al., 1979, Kandel, 2009). This type of habituation has been associated with a decrease in the number of synaptic vesicles in the presynaptic terminals of the sensory neurons following stimulation, leading to a reduction in the amount of the excitatory transmitter glutamate released into the synaptic cleft (Kandel, 2009). Conversely, if stimulation of the sensory neurons resulted in harm, then the withdrawal reflex would become sensitized and respond more vigorously to subsequent stimulation, due to the facilitated release of transmitter from the presynaptic terminals (Kandel, 2009). Finally, if a weak mechanical stimulus applied to the gill is paired with the delivery of an electric shock to the tail, associative memory will form as a result of activity dependent presynaptic facilitation of the same sensory neuron presynaptic terminals that can also undergo homosynaptic depression after habituation and presynaptic facilitation after sensitization (Kandel, 2009).

In these examples, behaviours, and even neuronal circuits themselves become altered and adapt to change the behavioural response of the organism as a result of learning.
These types of changes are often called plasticity and will be discussed in the following section.
1.2 Neuronal plasticity and the hippocampus

Neuronal plasticity is the term for the increasing or decreasing of communication signals in the nervous system. Synaptic plasticity is the process of synaptic alterations, including the insertion or removal of receptors in existing synapses, and an increase or decrease in pre-synaptic transmitter release (Berlucchi and Buchtel, 2009). Non-synaptic or intrinsic plasticity refers to changes in the functionality of axons, dendrites or the soma of a neuron, and is involved in the creation of new synapses adjacent to the synapse that is to be strengthened (Berlucchi and Buchtel, 2009). The following section will briefly review historical and current research of neuronal plasticity in both vertebrates and invertebrates.

The term ‘plasticity’ is now commonplace within neuroscience and increasingly used throughout the sciences in general, often to ambiguous ends. The term ‘plasticity’ has been historically used in the study of the brain to describe the ways in which the brain changes, adapts and reorganises itself (Berlucchi and Buchtel, 2009). It is generally thought that the brain is ‘mouldable’ and can be shaped like plastic.

One of the first uses of the term synapse was from Sir Charles Sherrington. Sherrington, who used the term to refer to the connection point between two neurons. He also posited that this connection point ‘ensures the unidirectionality of transmission’ between neurons (Sherrington, 1897, Sherrington, 1900, Sherrington, 1906). One of the earliest insights into how ‘synapses’ functioned was that electrical impulses would proceed through neurons and pass through the synapse of least resistance (Cooper, 2005). Both of these insights raised many questions and could be considered as the start of research in neuronal plasticity.

An important early contribution to this new field of thought was from Eugenio Tanzi in 1893 (Tanzi, 1893). Tanzi hypothesized that as a signal transduction occurred through a neuron, metabolic changes would occur that could result in increasing the size and/or volume of the connections between neurons. Additionally, he suggested that repeated stimulation of paired neurons would bring them physically closer together, and this would result in a decrease in resistance between the connections, therefore increasing the probability that the transduction would occur through that particular synapse. After repeated pairings an ‘associative bond’ would become established and this could house
a memory trace (Tanzi, 1893). Although today it is known that signal transduction does not follow a path of least resistance, these thoughts were later to be proved relatively accurate. Moreover, the resultant research into synapses and how they become modified was of the utmost importance to the field of modern learning and memory (Berlucchi and Buchtel, 2009).

Continuing with the hypothesis that local metabolism leads to increases in synaptic efficiency, Charles Child, a professor of embryology, theorised that all cells undergo development based on different levels of metabolic activity. Where high levels of metabolic activity acted as attractors. Child believed that learning and memory was a specialisation of a general process of cellular change (plasticity) and that classical conditioning could generate axonal growth between two separate pathways (Grossman, 1967, Child, 1924, Weidman, 1999, Cooper, 2005).

‘The process of cellular change’ was first called plasticity by Konorski. Konorski believed that the entire nervous system exhibited excitability, but only the cerebral cortex displayed plasticity, and that it could adapt to certain situations (Cooper, 2005, Konorski, 1948). Konorski and others at the time believed that recurring reverberatory activity between and within neurons was what initiated plasticity. And that this also required some structural change, and suggested that neurons could increase the number of connections between each other to increase the strength between neurons, particularly in the case conditioning (Konorski, 1948, Cooper, 2005).

Next to make a large impact on the fields of neuronal plasticity and learning and memory was Donald Hebb. Hebb was a student of the famous American psychologist Karl Lashley. Hebb’s work, written in “The Organization of Behavior: A Neuropsychological Theory” has received many citations in modern neuroscience and in particular to the field of learning and memory.

During his early career, Hebb found issue with lesion studies where portions of the brain were destroyed yet the ability to generate intelligence and learning were still intact. In this sense he was not convinced that certain brain processes were localised to certain portions of the brain, contrasting Lashley’s work. Hebb believed there was not enough evidence to suggest current, well received, localisation theories (Kandel, 2000, Hebb, 1949). Hebb found that with the development of intelligence and the strengthening of
connections between neurons, less neurons were needed, and the brain became more efficient. This led Hebb to his famous general theory of learning and memory, the ‘Hebbian learning rule’ that he postulated in 1949, which states; “When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased” (Hebb, 1949, Cooper, 2005). Later this became better known as ‘what fires together, wires together’, which in short argues that when two or more neurons are communicating together in repeated succession, they become stronger as a pairing, and allow for more efficient future communication between them. Like others before him Hebb too believed that structural changes occurred between synapses and that it was likely that there was an increase in the number of synaptic ‘knobs’ or an increase in the size of current synapses (Hebb, 1949, Cooper, 2005).

There has been evidence to show that “Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed” (Martin and Morris, 2002) (Cooper, 2005).

The development of research into the neurological underpinnings of memory diverted into two core directions, the study of explicit and implicit memory systems. The study of implicit memory largely utilised the relatively simple neuronal circuitry involved with simple reactions such as the gill-withdrawal in the sea slug Aplysia (Brunelli et al., 1976, Castellucci et al., 1978). By using ‘simple’ invertebrate models, that only contain a fraction of the neurons in the mammalian CNS, many of the mechanisms that we know today were discovered, and later also found in mammals. The study of explicit memory has commonly used the hippocampus of various rodents (rabbits, rats and mice) (Bliss and Gardner-Medwin, 1973, Bliss and Lomo, 1973, Schwartzkroin and Wester, 1975, Andersen et al., 1977, Kandel, 2009). This field received much attention and also resulted in many discoveries, many of which were also later replicated in invertebrates.

Despite the clear difference between explicit and implicit memory and also the differences between vertebrate and invertebrate nervous systems, the underlying logic and molecular biology are highly homologous between them (Kandel, 2009, Kemenes, 2013). Both types of memories function with similar temporal phases, more commonly
known as short-term and long-term memory. In both explicit and implicit memory across the species, protein synthesis is needed for the transition between STM and LTM, both in the mammalian hippocampus (Frey et al., 1993, Morris et al., 1982), and in *Aplysia* (Martin et al., 1997, Kandel, 2009). Moreover, both examples utilise similar molecular cascades, typically the PKA-MAPK-CREB signalling pathway (or the closest homolog within a given species) for converting STM to LTM, and both use various forms of additional plasticity to stabilise LTM (these topics will be discussed at length later in the chapter)(Bailey, 2008, Kandel, 2009, Sangha et al., 2003).

The study of explicit memory is arguably more complex than implicit memory. Human memory is extremely complex and uses various neural regions (Langston et al., 2010, Sweatt, 2010, O'Keefe, 2007). By reducing the complexity and studying one specific important region, such as the hippocampus, it is possible to slowly build up neurosciences knowledge of how memory functions.

1.2.1 The structure and function of the hippocampus

The hippocampal formation consist of the hippocampus, dentate gyrus, entorhinal cortex, subiculum and the pre-and-para subiculum. The hippocampus is situated within the temporal lobe of higher vertebrates, and is crucial for explicit memory storage (Andersen et al., 1980). The hippocampus itself is often referred to by its subsections, the Cornu Ammonis (CA) regions (1 – 4) (CA’s 1 and 3 are the major sections. CA2, although of reasonable size does not get the same attention as CA’s 1 and 3. CA4 is very small and of limited interest) and the dentate gyrus (Fig 1.2). The exact size and shape of the hippocampus differs slightly between species, however the ‘seahorse’ shape by which is name is derived is relatively consistent (Amaral, 2007). The most prevalent neurons in the hippocampus are the pyramidal neurons. Their cell body volumes and dendritic lengths differ depending on where they are located. For instance, in the dentate gyrus cell bodies are ~300μm² and have dendritic lengths of between 8 – 10 mm compared to pyramidal cells in the CA3 region where they are ~700μm² and 16 – 18 mm respectively (Ishizuka et al., 1995).
Figure 1.2. A diagram of the connections in the hippocampal formation. Innervation from the entorhinal cortex (EC) proceeds to the dentate gyrus (DG) via the perforant pathway (pp), or directly to the CA3 and CA1 regions via the pp. The dentate gyrus then processes and transmits too the CA3 via the mossy fibre (mf) connections. Within the CA3 there is a GABA-mediated recurrent collateral network that cycles transmission back into the CA3. The CA3 projects to the CA1 via the Schaffer collateral. To end the hippocampal loop the CA1 projects back to the entorhinal cortex either directly or via the subiculum (SUB). Figure from (Tsien, 1996).
The hippocampus’ extrinsic connections are predominately made by the entorhinal cortex and it is the entorhinal cortex that receives input from other cortical locations (Amaral, 2007). Once the hippocampus has received innervation from the entorhinal cortex the hippocampus itself acts as its main source of innervation. The CA3 region receives input from other CA3 neurons, these connections are often referred to as ‘commissural’ or ‘associational’ inputs. The identification of the CA3 recurrent collaterals supported the notion that the CA3 system functions as an associative attractor network that is used in working memory (McNaughton et al., 1986, Rolls, 1989, Kesner, 2007b, Bennett et al., 1994, Rolls, 1996, Nakazawa et al., 2004). One notable issue with so many interconnections is that the CA3 region is susceptible to seizure if its own regulatory inhibition processes are inhibited (Ben-Ari, 1985, Spruston, 2007, Amaral, 2007, Ishizuka et al., 1990, Li et al., 1994). Unlike most neural regions whose connections between two points are typically reciprocal, the hippocampus is largely unidirectional (Amaral, 2007). A typical ‘loop’ of the hippocampus (Fig 1.2) would start at the entorhinal cortex, innervate the CA3 either directly via the perforant pathway, or indirectly through the mossy fibre pathway via the dentate gyrus. From here the CA3 forms many connections to the CA1 and to itself (Ishizuka et al., 1990), where it forms associational connections and can stimulate even its own inhibition, therefore ceasing signal processing to the CA1 (Amaral, 2007). The CA1 then projects back onto the entorhinal cortex (Naber et al., 2001). Until recently it was generally considered that all regions project back to the entorhinal cortex, however it is now known that it is only the CA1 region (Amaral, 2007).

Understanding hippocampal anatomy is relatively straightforward. Understanding how this fits with the many theoretical functions is much more complex. However, it is clear the hippocampus is very important to correct memory functioning (Annese et al., 2014, Kandel, 2009, Vann and Albasser, 2011, Sweatt, 2010, Langston et al., 2010). Many of the current models of hippocampal function have been developed from Marr’s original theories (Marr, 1971, Willshaw et al., 2015). It was hypothesised that the hippocampus needed to either have greater information storage potential and/or be highly efficient (Langston et al., 2010). This was advanced upon with additional computational studies suggesting that the hippocampus is required for the rapid encoding of stimulus conjunctions, and their recall (Rudy and O’Reilly, 2001, O’Reilly and Rudy, 2000).

Its individual sub regions have also shown specific specialties. For instance, The CA3 region is important in the acquisition and encoding of short-term spatial memory (Kesner,
and there is strong evidence it is crucial in encoding novel information quickly (Nakazawa et al., 2002, Kesner, 2007b). Another important feature of the hippocampus is its role in pattern completion (Stark, 2007). Pattern completion allows for the retrieval of an associated memory, even with just a portion of the original stimuli. This is hypothesised to be a function of the CA3 collateral network (Langston et al., 2010). In support of this hypothesis, mutant mice with a deletion of the CA3 specific glutamate NR1 were impaired in performance of a Morris water maze when their cues were removed (Nakazawa et al., 2002). A specific role for the dentate gyrus as a pattern separator has also been hypothesised (McNaughton et al., 1986, O'Reilly and Rudy, 2001). However, others feel that its role is more involved in fine scale spatial pattern separation and not non-spatial pattern separation (Rolls and Kesner, 2006, Kesner, 2007a). As in the investigation of the CA3, mutant glutamate NR1 deficit mice specifically in the DG did not support its role in fine scale pattern separation (Langston et al., 2010). Therefore it is proposed that the CA3 matches stimuli projecting from the EC with previous stimuli, and the DG separates and encodes novel stimuli projecting from the EC (Langston et al., 2010). A role for CA1 is currently less defined, though it has been proposed that it may compare the processed input from the CA3 with the original input from the EC, as it has both connections (Langston et al., 2010). Others have suggested that the CA1 adds temporal context, adding a temporal factor to the retrieved paired associations from the CA3 region (Rolls and Kesner, 2006, Wallenstein et al., 1998).

**1.2.2 Long-Term Potentiation**

The hippocampus contains three distinct neuronal pathways; perforant, mossy fibre and Schaffer collateral (Fig 1.2 and 1.3). Using anesthetized rabbits it was discovered that stimulation of any of these three pathways, with a short train of electrical stimulation, resulted in an increase in response to subsequent stimulation (an increase in fEPSP amplitude) in the subsequent pathway. This effect was termed long-term potentiation (LTP) (Bliss and Lomo, 1973). Shortly after this finding, it was confirmed that LTP could be induced in awake, freely moving rabbits (Bliss and Gardner-Medwin, 1973) and rats (Douglas and Goddard, 1975). LTP can be described as 'a rapid and persistent synaptic enhancement' (Bliss and Lomo, 1973), persistent stating that it outlasts any other form of potentiation at the time such as facilitation or post-tetanic potentiation (PTP) (Brown et al., 1988). LTP is not uniform throughout the brain, even within the hippocampus LTP has several forms. LTP is associative in both the perforant and Schaffer collateral pathways and non-associative in the mossy fibre pathway (Bliss and Collingridge, 1993, 2007b).
LTP has been found in hippocampal slices of several different species (Schwartzkroin and Wester, 1975, Alger and Teyler, 1976, Yamamoto and Chujo, 1978, Andersen et al., 1977, Lynch et al., 1977) and is thought to be the underlying mechanism involved in memory acquisition and formation, and it is strongly linked with the acquisition of spatial memory (Pyapali et al., 1999, Steele and Mauk, 1999, Nguyen et al., 2000).

It has been shown that in the hippocampus, LTP has both an early and late phase. A single train of stimuli can produce the early phase of LTP, E-LTP, this phase lasts around 1 – 3 hours and is independent of protein synthesis (Bolshakov et al., 1997). Multiple trains of stimulation produce late phase LTP, L-LTP, this phase lasts 24 hours or more and requires protein synthesis (Frey et al., 1993, Abel et al., 1997) (Bolshakov et al., 1997).

Early research indicated that the induction of LTP modifies neuronal properties such that the stimulation necessary for subsequent neuronal signalling is reduced, resulting in a more attuned pathway (Abraham et al., 1987, Andersen et al., 1980, Taube and Schwartzkroin, 1988). After LTP is induced, subsequent EPSP’s display a greater output, and is achieved by the insertion or modification of post-synaptic voltage-dependent receptors (Daoudal et al., 2002, Wang et al., 2003, Xu et al., 2005). What causes the increase in EPSP’s associated with LTP was of much debate. Malinow showed that during LTP synaptic variability and failures decrease, suggesting that excess transmitter is released (Malinow and Tsien, 1990). Nicoll found that Schaffer collateral LTP was associated with an increase in AMPA receptors, with no change in NMDA receptors. This provided the first evidence that Schaffer collateral LTP is induced and expressed post-synaptically (Kauer et al., 1988, Kandel, 2009).

LTP’s counterpart – Long-term depression (LTD) was discovered by Gary Lynch’s group. Instead of firing high-frequency bursts of stimulation, they fired low-frequency bursts and found that this reduced the EPSP activity of the targeted area within the hippocampus and induced a depression. Moreover, they continued to show that more ‘natural’ patterns of stimulation were able to produce LTP, such as theta bursts (Larson and Lynch, 1986, Cooper, 2005, Stevens and Wang, 1994). Around the same time, Collingridge et al showed that NMDA receptors were crucial in hippocampal LTP. They found that NMDA could trigger LTP, conversely, by using AP5, a NMDA antagonist, they could block the induction of LTP (Collingridge et al., 1983b, Collingridge et al., 1983a, Huganir and Nicoll, 2013). This important finding lead the way for subsequent research into LTP, and it is
now thought that many of the molecular mechanisms involved are now known. The following section will outline the core molecular mechanisms involved in the induction and maintenance of LTP, with particularly attention paid to the hippocampal CA3 – CA1 region.

Before the mechanisms of LTP are outlined, it would be useful to summarise the whole process of LTP induction in brief, taking the CA3 – CA1 Schaffer collateral pathway as an example. As an action potential reaches a synapse and results in the depolarisation of the post-synaptic membrane Na$^{2+}$ influxes through post-synaptic AMPA receptors (Malinow and Miller, 1986, Wigstrom et al., 1986, Chater and Goda, 2014). The Na$^{2+}$ influx causes a depolarisation and expels the Mg$^{2+}$ block in the pore of the post-synaptic NMDA receptors (Mayer et al., 1984, Nowak et al., 1984). At the same time, glutamate binds to the post-synaptic NMDA receptor (Collingridge et al., 1983b), when this occurs at the same time Ca$^{2+}$ can flow through the NMDA receptor and into the post-synaptic cell (Nicoll et al., 1988). Once inside Ca$^{2+}$ is involved with various plasticity related changes, such as binding with calcium-dependent calmodulin kinase II (CaMKII) (Huganir and Nicoll, 2013, Tsien, 2000).

Although LTP is a naturally occurring phenomenon (O'Keefe, 2007, Stark, 2007, Sweatt, 2010), it can be artificially induced by using brief bursts of electrical stimulation termed high-frequency stimulation (HFS) (Bliss and Gardner-Medwin, 1973, Bliss and Lomo, 1973, Fujii et al., 2008, Bortolotto et al., 2011). A typical procedure would be a single 100 Hz stimulation at around half the voltage needed to elicit a maximal EPSP (Bortolotto et al., 2011). This technique has been extensively used and has been shown to induce robust LTP (Huang and Kandel, 1994, Bortolotto et al., 2011). Different induction techniques result in different types of LTP. As an example a single HFS produces LTP independent of PKA (Bortolotto and Collingridge, 2000, Sajikumar et al., 2007). However, if HFS is repeated PKA becomes incorporated in LTP (Frey et al., 1993, Ramachandran et al., 2015). It is important however to understand that LTP is an artificial form of synaptic plasticity, and that this may only tell us so much of the story of the real mechanisms behind synaptic plasticity in vivo. That aside, as you will come to read there is a full body of compelling evidence linking it to both real-life models in vivo and therefore positioning itself at the leading theory for the building blocks of learning and memory (Tsien, 2000).
Figure 1.3. A diagram of LTP induction. (A) A cartoon of the hippocampal slice structure. The two stimulation electrodes can be seen placed towards the centre of the Schaffer collateral pathway, with a recording electrode positioned in the CA1 pyramidal cell body layer. When the two stimulation electrodes perform a high frequency stimulation the EPSP slope% immediately increases (B). With multiple stimulation LTP increases. LTP refers to a persistent change in output, in the example given the late LTP trace shows a large increase in output consistently for three hours. Image from Stark (2007).
Figure 1.4. The mechanisms involved in long-term synaptic transmission in LTP. (A) PKA activates CREB which turns on the genes that are needed for long-term synaptic restructuring. (B) A fluorescent dyed CA1 pyramidal neuron. (C) The growth of new dendritic spines. Image from Purves (2004).
1.2.1.1 The role of NMDA and AMPA receptors

As mentioned previously, the NMDA receptor was found to be involved with the induction of LTP in the hippocampus (Collingridge et al., 1983b, Coan et al., 1987). The glutamatergic NMDA receptor is a heterotetradimer that must contain two GluN1 subunits with two GluN2 and/or GluN3 subunits (Vyklicky et al., 2014). In the Schaffer collateral pathway the NMDA receptor facilitates LTP by influxing Ca\(^{2+}\) into the post-synaptic neuron (Nicoll and Malenka, 1999, Collingridge et al., 1983b, Bliss and Collingridge, 1993). Others have shown the importance of NMDA receptor activation to the induction of LTP. Brown (Brown et al., 1988) used AP-5 to block NMDA receptors and replicated the finding that it blocked LTP (Collingridge et al., 1983b, Collingridge et al., 1983a). Although most types of LTP are induced by the activation of NMDA receptors, LTP is not a single receptor process. Once NMDA receptors have been activated, mGluR5 and the adenosine A2A receptor both need to be activated (O'Keefe, 2007, Sweatt, 2010). NMDA receptor activation allows for an influx of Ca\(^{2+}\) that activates specific G-proteins which are coupled to the intracellular portion of the post-synaptic NMDA receptor. An influx of calcium also signals for a further Ca\(^{2+}\) to be released from the intercellular store through the interaction with IP\(_3\) (Harney et al., 2008). There is also evidence of NMDA receptor independent LTP. In certain circumstances LTP can be formed by the use of use voltage-gated calcium channels (VGCC) and/or ryanodine receptors (Raymond and Redman, 2002, Grover and Teyler, 1990), although these techniques have not nearly been as well studied as NMDA receptor dependent LTP.

Another glutamate receptor that is important in neuronal plasticity and LTP are AMPA receptors, or \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. AMPA receptors are tetrameric, cation-permeable ionotropic glutamate receptors (Beneyto and Meador-Woodruff, 2004). When synaptic glutamate binds to an AMPA receptor, the central pore opens and allows for the influx of Na\(^{2+}\) and the efflux of K\(^{2+}\). With enough AMPA receptors the influx of Na\(^{2+}\) can reach the threshold level for depolarisation of the post-synaptic membrane to occur (Chater and Goda, 2014). AMPA receptors are responsible for the majority of fast, excitatory conduction in the brain. Therefore the regulation of AMPA receptors is crucial to the correct functioning of learning and memory (Hanley, 2014).

AMPA receptors play various roles in synaptic plasticity and LTP. AMPA receptors can be rapidly inserted into the post-synaptic membrane and increase glutamate transduction in that particular pathway (Shi et al., 1999, Carroll et al., 1999, Nicoll et al.,
The insertion of AMPA receptors into the post-synaptic membrane was shown by staining the AMPA receptors and then activating the NMDA receptors. By using florescent microscopy the AMPA receptors could be tracked and were shown to be trafficked to the post-synaptic membrane, and in greater numbers than originally thought (Shi et al., 1999). They are trafficked to the membrane by a repeated process of exocytosis, endocytosis and endosomal recycling (Hanley, 2014). Additional AMPA receptors increases both the chance of successful glutamate binding, and the total amount of Na\(^+\) and K\(^+\) ions that can influx within a single pre-synaptic transmitter release. Therefore increasing the chance that the post-synaptic membrane becomes depolarised. An increase in post-synaptic AMPA receptors is sufficient for the early phase of LTP and therefore possibly sufficient for STM. However, for the consolidation of a memory to occur there needs to be new protein synthesis and up-regulation of certain associated genes (Reymann and Frey, 2007). In unusual circumstances there are AMPA subunit compositions that allow for the influx of Ca\(^{2+}\), therefore in these cases AMPA receptors could play an NMDA independent role in synaptic plasticity (Chater and Goda, 2014).

### 1.2.1.2 The role of Calcium and CaMKII

Calcium plays various roles once it has entered into the post-synaptic neuron. It can bind with calcium-calmodulin protein kinase II (Malenka et al., 1989, Malinow et al., 1988), and/or PKC (Routtenberg, 1986, Malinow et al., 1988, Kandel, 2009, Huganir and Nicoll, 2013). CaMKII is one of the most common proteins found in the CNS (Lisman et al., 2002, Zhang and Lisman, 2012), and after calcium enters through a post-synaptic receptor it often binds to calmodulin, this newly formed complex binds to CaMKII and allows it to both autophosphorylate and become autocatalytic. These two mechanisms both enable the CaMKII to remain active beyond the limitation of calcium entry (Hell, 2014, Lisman et al., 2012). Among other functions, CaMKII acts to phosphorylate certain target proteins, including the GluA1 subunit of the AMPA receptor (Barria et al., 1997, Mammen et al., 1997, Chater and Goda, 2014). The involvement of CaMKII with LTP has been shown to be very significant. By injecting hippocampal slices with EGTA, a calcium chelator, LTP can be blocked. However, other areas free of EGTA can still induce LTP (Lynch and Baudry, 1984). It was later shown that calcium influx itself was needed for LTP to occur (Malenka et al., 1988). In a series of experiments where the CaMKII gene was mutated, it was shown that little to no side-effects were evident due to the mutation alone. However, it did inhibit LTP in the CA1 region of the hippocampus,
even though the NMDA receptors were unaffected by the mutation (Silva et al., 1992a, Silva et al., 1992b). Moreover, it was later shown that if CaMKII itself was directly injected into the CA1, this mimicked the effect of CA1 LTP (Lledo et al., 1995), although this effect was diminished if it was injected after LTP was already induced (Lledo et al., 1995). Finally, when dyed CaMKII were inserted into hippocampal neurons via a virus, the concentration of CaMKII was increased at the post-synaptic density (PSD) immediately after LTP was induced (Shen and Meyer, 1999).

1.2.1.3 The role of the Protein kinases A and C and CREB

There are other kinases involved in hippocampal LTP such as protein kinase A and C (PKA, PKC), along with CREB these molecules are instrumental in the transition between short-term and long-term memory consolidation (Michel et al., 2008, Kandel, 2012, Waltereit and Weller, 2003). By utilising a transgenic mouse deficient in the catalytic subunit of PKA, it was revealed that the late phase of LTP was unable to be produced. Demonstrating that PKA has a role in the transition from E-LTP to L-LTP (Abel et al., 1997, Abel et al., 1998). PKC has been shown to be involved with memory even later than PKA. A PKC isoform known as PKM-ζ is autonomous and crucial for the maintenance of LTM. If PKM-ζ is interfered with, a memory can be affected days or weeks after its original formation (Serrano et al., 2008). Mice with mutations in CREB-1 displayed a severe deficit in long-term spatial memory and LTP. As with PKA, the early phase of LTP/memory was normal, however the late phase was impaired (Silva et al., 1992a, Silva et al., 1992b, Bourchuladze et al., 1994, Abel et al., 1997).

1.2.1.4 LTP and spatial memory

The link between hippocampal LTP and spatial memory has been extensively studied. Richard Morris in 1986 was the first to experimentally show the link between LTP and spatial memory (Morris et al., 1986). Morris found that NMDA receptors needed to be activated for functioning spatial learning to occur. It was also shown that when NMDA receptors were blocked, LTP cannot occur and the animal could not form spatial memories (Morris et al., 1982). By selecting for certain genes it is possible to compare the effects of deletion or mutations in both the hippocampi of live animals and hippocampal slices. By doing so it was demonstrated that knocking out the genes coding for the kinases CaMKII and fyn disrupted spatial memory and LTP even though PPF and
PTP were normal (Silva et al., 1992a, Silva et al., 1992b, Grant et al., 1992). Using a NMDA receptor mutant mouse line, NMDA receptors were only expressed in the pyramidal neurons of the CA1. These mutants showed no obvious abnormalities or any deficit in non-spatial memory, but they did show impaired spatial memory. This demonstrated the importance of hippocampal NMDA receptors for spatial memory (Tsien et al., 1996a, Tsien et al., 1996b). Studies using the Morris water maze showed that the NMDA antagonist AP-5 impaired spatial memory performance in the water maze (Morris et al., 1986). In parallel to this it was shown that saturation of LTP before training in a spatial memory task, impairs spatial learning (McNaughton et al., 1986). Similar studies have also shown LTP to be essential for spatial memory in the Barnes maze (Barnes, 1979).

Although most of the research investigating LTP are in small mammals, in part due to the need of a recently culled subject, there is some work in humans. This work allowed for the confirmation that the mechanisms are between humans and mammals are homologous. For example, in a human study of synaptic plasticity, the hippocampus was found to show NMDA dependent hippocampal LTP, and that it was readily induced (Beck et al., 2000). This result was replicated in the human temporal lobe, where even the same electrical stimulation protocols (HFS) achieved the same results as in rodents. Moreover, drugs such as the NMDA receptor antagonist AP-5 blocked the induction of LTP in humans, as it does in mammals (Chen et al., 1996, Clapp et al., 2012, Collingridge et al., 1983a).

### 1.2.3 Invertebrate learning and memory: Long-term facilitation

Invertebrates have been often used throughout neuroscience’s history due to their relative simplicity. They have many times fewer neurons than vertebrates and typically exhibit less complex behaviours (Menzel et al., 2013, Benjamin, 2008). Moreover, they are cheap to house and experiment upon and it reduces the use of experimenting on higher mammals, which some find objectionable. Historically, invertebrates have been used to study implicit memory, although many of the molecular mechanisms involved in simple reflexive learning are also involved in more complicated explicit mammalian systems (Sweatt, 2010, Kemenes, 2013) (see Fig 1.5).
The study of the connections between sensory neurons and motor neurons of gill-withdrawal in *Aplysia* allowed for investigation of the short-term facilitation seen in sensitization of the gill-withdrawal reflex (Brunelli et al., 1976, Bailey et al., 2015). In the sensitization of the gill-withdrawal reflex several synaptic sites undergo changes, both short-term and long-term sensitization are due to increased transmitter release. Serotonin released from the tail sensory neurons after 1 stimulation produces short-term facilitation, after 5 or more stimulation it can lead to long-term facilitation (Kandel, 2000). One of the early studies linking neuronal plasticity with sensitization in *Aplysia* showed that the sensory neurons increased the number of presynaptic terminals after sensitization, and reduced them after habituation (Bailey and Chen, 1988, Bailey and Chen, 1983, Bailey et al., 2015). The molluscan models of memory do not undergo LTP like in the mammalian hippocampus, however they do undergo long-term facilitation (LTF), which shares many of the same important features (Kandel, 2012, Kandel, 2009, Kandel, 2000). For instance, it has been shown that even if cAMP or PKA are injected directly into the sensory neurons, the synapse is enhanced (Castellucci et al., 1980). The binding of cAMP to PKA to create thepersistently activated form of PKA is essential for LTF in *Aplysia*, and PKA plays a key role in the formation of LTM and synaptic plasticity (Schacher et al., 1988, Abel et al., 1997, Muller, 2000, Davis, 2005, Michel et al., 2008). Moreover, if this catalytic subunit of PKA is directly injected into the sensory neurons, then this is sufficient for LTF (Chain et al., 1999). Moreover, PKA is involved in single-trial learning, and is increased shortly after classical conditioning. It is both involved with the early phase of memory (~6h) and a prolonged increase involved with late phase memory (~24h) (Michel et al., 2008). These mechanisms are highly conserved and leads to the signalling for protein synthesis to consolidate LTM (Selcher et al., 2002, Roberts and Glanzman, 2003, Barco et al., 2006, Schwaerzel et al., 2007, Michel et al., 2008). In this example adenylyl cyclase acts as a coincidence detector because it is awaiting both the input from the sensory neuron and the resultant ca^{2+} influx, as well as the depolarisation arising from the tail poke (Kandel, 2000) (see *Fig 1.5* for comparison between *Aplysia* and the hippocampus).
Fig. 1.5 Mechanisms involved with the formation of Long-term memory in Aplysia (A) and the hippocampus (B). Both are initiated from a neurotransmitter-receptor binding leading to activation of the cAMP-PKA-MAPK-CREB pathway. This activation results in the trafficking of AMPA receptors into the synapse as well as de novo protein synthesis from the nucleus, which is necessary for synaptic growth.
As in mammals, the transfer of STM to LTM, and STF to LTF was found to be CREB dependent (Abel et al., 1998). Conversely CREB-2 has been shown that when overexpressed it blocks LTF in *Aplysia* (Bartsch et al., 1995, Yin et al., 1994). However, if CREB-2 is removed from the system, then a single stimulus that would usually only lead to STF can lead directly to LTF (Yin et al., 1994). Although LTF has been studied in much greater detail in molluscs, LTP has also been shown in *Aplysia*. High-frequency stimulation induced LTP in the sensorimotor synapse, which was shown to involve a post-synaptic voltage dependent mechanism, mirroring the LTP seen in the hippocampus (Lin and Glanzman, 1994).

At first it may feel unreasonable to compare the complex behaviours of a human or mouse to the humble snail, and in most cases this is correct. Although evolution has increased the diversity within species the conservation of memory, and the mechanisms underlying memory have been highly conserved (Tascetta et al., 2015), also invertebrates are easier to experimentally manipulate and therefore make excellent tools for research into memory. For instance, both the mouse and *Lymnaea* animal models require the use of the cAMP-PKA-MAPK-CREB molecular pathway which consolidates STM/E-LTP to LTM/L-LTP (Ribeiro et al., 2005, Waltereit and Weller, 2003, Bailey et al., 2015).
1.3 How alcohol affects the brain

Alcohol has been used widely throughout human history, and it should come as no surprise that it has received a lot of attention from scientists and thinkers throughout that time. However, although the effects that alcohol has on human behaviour is relatively well known, the interaction it has on the neuronal mechanisms underlying these behaviours is much less understood (Davies et al., 2003a). Therefore in more recent times many have strived to achieve a better understanding of this prolific and perhaps underestimated substance.

Once drunk, ethanol is absorbed into the blood. The stomach absorbs 20% and the rest is absorbed by the upper intestine. The maximal concentration varies, but it typically in the range of 30 – 90 minutes after the last drink (Julien, 2011). Impaired ability begins at around BAC of 0.01g%, however at between 0.04 and 0.08g% there is significant impairment of judgement and reactions (Ridderinkhof et al., 2002).

One of the major problems when attempting to understand the actions of ethanol is that ethanol has no particular molecular target and has a low affinity to bind to proteins (Harris et al., 2008). Ethanol has direct inhibition of some subunit conformations of nicotinic acetylcholine (nACh) receptors and GABA-A receptors (Hunt, 1983). This results in varied effects depending on which subunits are present and the concentration of ethanol (Aguayo et al., 2002, Cardoso et al., 1999, Davis and de Fiebre, 2006, Roberto et al., 2003, Huettner and Bean, 1988). However, ethanol indirectly interacts with many types of receptors, including NMDA (Hoffman et al., 1990, Huettner and Bean, 1988), serotonin (LeMarquand et al., 1994, Sari, 2013), Glycine (Sebe et al., 2003, Lovinger and Roberto, 2011, Aguayo et al., 1996), and AMPA (Dildy-Mayfield and Harris, 1992, Moykkynen et al., 2003). Ethanol often acts on these receptors, to modulate the binding of other neurotransmitters (Tonner and Miller, 1995, Welsh et al., 2009), alter the probability of a channel opening (Zhou et al., 1998), and even increase or decrease the probability of presynaptic transmitter release (Roberto et al., 2006, Dolganiuc and Szabo, 2009).

One of the most common targets for ethanol are the GABA receptors. This action is believed to be the cause of the drowsiness general drunken demeanour that occurs during ethanol intoxication in humans (Hunt, 1983, Lovinger and Roberto, 2013). This is supported by GABA agonists producing similar physiological effects (Olsen et al., 2007).
Generally, increases in GABA and glycine activity produce greater levels of inhibition in neurons (Lovinger and Roberto, 2011). Ethanol can increase the amplitude and/or duration of the inhibitory post-synaptic potentials (IPSP’s) resulting from GABA-A and Glycine receptors (Sebe et al., 2003). In particular ethanol appears to potentiate GABA receptors containing α-β-γ subunits and α-4 or α-6 that are formed with β and δ subunits (Olsen et al., 2007, Lovinger and Roberto, 2011). Ethanol also has a potentiating effect on glycine-dependent chloride channels. Where chloride receptors that contain the α-1 subunit appear to be more sensitive to ethanol potentiation than the α-2 subunit (Davies et al., 2003b, Mihic et al., 1997, Mascia et al., 1996).

Ethanol has also been shown to potentiate 5-HT3 receptors (Machu and Harris, 1994). Serotonin receptors have little role in acute ethanol intoxication, however they have involvement in ethanol-reward and alcoholism (Sari, 2013). Serotonin neurotransmitter can mediate voluntary ethanol use in individuals, typically, increases in serotonin result in increased ethanol intake and vice versa (LeMarquand et al., 1994, Sari, 2013).

Ethanol at relative intoxication levels has been shown to play in inhibitory role in NMDA receptors function (Criswell et al., 2003, Hoffman et al., 1989, Lovinger et al., 1989, Allgaier, 2002). Although NMDA receptors are all affected by ethanol certain NMDA-subtypes are more sensitive than others (Lovinger and Roberto, 2011, Allgaier, 2002), with NR1/2A and NR1/2B subunits particularly sensitive to the effects of ethanol, whereas NR1/2C less so (Masood et al., 1994, Chu et al., 1995). Similar to the effect on NMDA receptors ethanol also inhibits AMPA receptors (Dildy-Mayfield and Harris, 1992, Moykkynen et al., 2003). However there has not been any reported differences in AMPA receptor subunit sensitivity to ethanol (Lovinger and Roberto, 2013). However, ethanol appears to have a preference for NMDA receptors over AMPA receptors (Lovinger et al., 1989, Lovinger, 1995, Lovinger and Roberto, 2011).

Ethanol also has weak direct impact on G-protein coupled receptors and their resultant cascades (Lovinger and Roberto, 2011), such as AC, cAMP and CREB (Luthin and Tabakoff, 1984, Rabin and Molinoff, 1981, Asyyed et al., 2006). AC’s multiple isoforms show different sensitivities towards ethanol, AC7 is the most sensitive, 2 – 3 times more so than AC2, AC5 and AC6, AC3 has been shown to have no effect to ethanol (Anis et al., 1983, Huettner and Bean, 1988).
1.3.1 Defining binge drinking

Seeing how ethanol displayed such complicated interactions with nervous tissues at varied concentrations of acute ethanol doses, it should come as no surprise that understanding the effects that human drinking is also very complicated. The varied drinking patterns, such as binge drinking or chronic alcoholism add new variables to an already highly variable study. Nonetheless, these drinking patterns are important to understand as they are highly prevalent and could therefore affect a large number of people.

Alcohol has a strong effect on behaviour and physiology after the blood alcohol concentration (BAC) reaches around 0.08% or greater (Ridderinkhof et al., 2002, Dolganiuc and Szabo, 2009). It is the elevated BAC that is responsible for the effects of alcohol and not the route of administration (Dolganiuc and Szabo, 2009). The NHS uses a research driven definition for binge drinking “Researchers define binge drinking as consuming eight or more units in a single session for men and six or more for women” (NHS, 2015). The US National Institute on Alcohol Abuse and Alcoholism (NIAAA) defined a ‘binge’ as “a pattern of drinking that produces BAC’s greater than 0.08%” (NIAAA, 2004). In human adults this is the equivalent of 5 standard drinks in males, 4 in females, within a 2 hour period, this is often called the 5/4 paradigm (Crabbe et al., 2011, McBride et al., 2014). Furthermore, a binge drinker can be characterised by the performing repeated consumptions of large amounts of alcohol within a short duration, followed by a period of abstinence (Townshend and Duka, 2005, Stephens and Duka, 2008). This pattern of binge alcohol abuse seems to be of particular relevance, as it takes in account the rapid increase in BAC’s, and also appears result in more detrimental outcomes (Campanella et al., 2013, Petit et al., 2014).

Within the alcohol use spectrum binge drinking can be considered between chronic alcoholism and moderate alcohol use. Binge drinkers differ from alcoholics in that they do not suffer from dependency, have periods of abstinence, and are able to refuse drinks in self-administration tests (Courtney and Polich, 2009, Courtney and Polich, 2010, Oei and Morawska, 2004).
1.3.2 The effects of binge drinking in humans

Binge drinking, across a broad age range has been linked with; an increased risk of mood disorder (Okoro et al., 2004), aggression (Shepherd et al., 2006), heart disease, high blood pressure and type II diabetes (Fan et al., 2008), deficits in language (Moss et al., 1994), increases in glutamate concentration (4–5 times the normal level) (Ward et al., 2009), abnormalities in white matter (Jacobus et al., 2009, Chung and Clark, 2014), smaller cerebellar volumes (Lisdahl et al., 2013), higher activity in the left amygdala and insula during a decision making task (Xiao et al., 2013), poorer motor performance in certain tasks (Fogarty and Vogel-Sprott, 2002, Courtney and Polich, 2009), deficits in inhibitory control (Lopez-Caneda et al., 2014a), and in some cases frontal deficits similar to those found in Korsakoff alcoholics (Hartley et al., 2004, Courtney and Polich, 2009, Spear, 2015). Binge drinking has also been shown to affect cognitive abilities such as; retrieval of verbal and non-verbal information, visuospatial functioning (Brown et al., 2000, Hanson et al., 2011) which is greater in females (Squeglia et al., 2009), deficits in episodic memory and planning (Hartley et al., 2004), spatial working memory and pattern recognition (Tapert et al., 2004b, Weissenborn and Duka, 2003, Townshend and Duka, 2005), impairment in frontal lobe functioning (Scaife and Duka, 2009, Crews et al., 2007) (Squeglia et al., 2011), a reduction in executive functioning (Moss et al., 1994, Giancola et al., 1996, Hartley et al., 2004), reduced decisive decision making (Goudriaan et al., 2007, Thiele and Navarro, 2014), impairment of STM (Chait and Perry, 1994), impaired cerebellar function (Squeglia et al., 2011), impaired prospective memory (Heffernan et al., 2010), and impairment in temporal lobe function with females showing additional impairment in dorsolateral prefrontal cortex function (Scaife and Duka, 2009, Squeglia et al., 2011). These lists however, come largely from correlation studies, and although they draw conclusions from strict statistics, they are also open to interpretation to other factors such as general physical or mental health issues, the use of other drugs and largely having used populations from within universities.

There is also growing evidence and attention towards the effects of repeated withdrawal from ethanol, like the durations between binges, producing some of the long-term deficits associated with binge drinkers (Glenn et al., 1988, Parsons and Stevens, 1986, Stephens et al., 2005, Courtney and Polich, 2009). In humans it was found that the number of withdrawal periods (24 h abstinence) correlated with the extent of the memory deficit (Hunt, 1993, Glenn et al., 1988). This has been replicated in animal studies, where it was clearly shown that ethanol binges, and multiple withdrawals from ethanol produced

Many of the above studies have been replicated in animal models. As accurate social effects of binge drinking are difficult to model in animals, much of the research has been focussed towards neurophysiological effects. Studies have shown that binge like ethanol treatments can lead to neurodegeneration in; corticolimbic regions that are linked to the deficits in learning and spatial memory (Jarrard, 1993), olfactory bulb, piriform cortex, perirhinal cortex, entorhinal cortex and the hippocampus (Collins et al., 1996, Corso et al., 1998, Crews et al., 2000, Zou et al., 1996).

This thesis is not wholly interested in the effects of chronic alcoholism and addicted drinking. Alcoholism is a complex disease in its own right, however there is some crossover and very heavy binge drinking and heavy ethanol treatment protocols may better reflect chronic drinking. Therefore it is important to distinguish between the two.

### 1.3.3 The effects of chronic ethanol and how it differs from binge drinking

Chronic alcohol use is associated with impairments in cognition and memory and is often referred to as ‘alcoholic dementia’ (Oslin and Cary, 2003, Zorumski et al., 2014). As chronic alcohol use, or alcoholism is a long-term disorder it is often hard to characterise changes in cognition. However, studies suggests that roughly 50 - 75% of alcoholics develop some form of cognitive dysfunction (Vetreno et al., 2011, Parsons and Nixon, 1998). With most severe cognitive deficits from alcoholism come from Korsakoff’s syndrome, who suffer from sever prefrontal deficits (Acheson et al., 2013). Other such deficits associated with chronic alcohol consumption include; executive functioning, decision making and memory (Dom et al., 2006, Cacace et al., 2011), significant motor deficits (Teixeira et al., 2014), weight and volume reductions in the cerebral cortex, hippocampus and cerebellum (Harper and Blumbergs, 1982, Harper, 2009, Teixeira et al., 2014), neuronal loss in the hippocampus and cortex (Farr et al., 2005), varied effects on gene regulation (McClintick et al., 2015), as well as general frontal lobe dysfunction (Loeber et al., 2009).
Prolonged ethanol use leads to ethanol dependence. Dependence leads to neuroadaptive changes in the brain that can lead to further drinking, despite loss of health (Griffin, 2014). Part of these changes is the upregulation of glutamatergic activity (Gass and Olive, 2008, Griffin, 2014). Chronic drinking upregulates synaptic NMDA receptors, but has no effects on non-synaptic NMDA receptors (Carpenter-Hyland et al., 2004). The increase in extracellular glutamate has been identified in numerous locations including the nucleus accumbens, dorsal striatum, and the hippocampus (Dahchour and De Witte, 2003, Dahchour et al., 2000, Rossetti and Carboni, 1995), additionally MRI revealed an increase in glutamate in the PFC (Hermann et al., 2012) and basal ganglia (Zahr et al., 2009).

Generally the where the symptoms are shared between binge drinkers and alcoholics they are much more severe in alcoholics (Griffin, 2014, Acheson et al., 2013). However, binge drinking is much more wide-spread and has a much greater prevalence in adolescents than alcoholism (Loeber et al., 2009, Leeman et al., 2010). Highlighting the need to fully understand how binge-like drinking effects both the developed and developing brain.

1.3.4 Why investigate binge drinking in adolescence?

Adolescence is a developmental period where the brain is undergoing increased levels of change including synaptic pruning, cortical restructuring and maturation (Giedd, 2004, Giedd, 2008). During adolescent development certain behavioural changes occur that are essential for the acquisition of skills that will be needed in adulthood (Blakemore and Choudhury, 2006), such as intelligence (Shaw et al., 2006) and behavioural control (Ernst et al., 2009). Rodents undergo a similar developmental period during adolescence, which is defined by changes in hormones, behaviour and sexual development (Maldonado-Devincci et al., 2010). Oddly, although human adolescents are stronger, faster and more resistant to disease than children, the death rate is 200% higher (Dahl, 2001). This could be in part due to adolescents developing behaviours such as increasing the level of social interaction, elevating physical activity, risk-taking, impulsivity, and novelty seeking (Ernst et al., 2009, Spear, 2000, Spear, 2015).

Human adolescents are known to consume high levels of ethanol (Bates and Labouvie, 1997, Maldonado-Devincci et al., 2010, Spear, 2015), interestingly this is also true during
some rodent strains adolescence (Doremus et al., 2005, Griffin, 2014). The combination of developing social attributes, peer-pressure, and alcohol’s low sedative response in adolescents may result in dangerous behaviours such as drug and alcohol abuse (Chambers et al., 2003) with patterns of alcohol consumption analogous to binge drinking (Silveri and Spear, 1998, Varlinskaya et al., 2001, Spear, 2015, Spear, 2013).

1.3.5 The effects of binge drinking and binge-like ethanol treatments during adolescence

Neurophysiological studies during adolescence have shown that human adolescents show a consistent linear rise in cortical white matter volume, a fall in frontal and parietal grey matter volume during the periods of late adolescence and early adulthood (Fein et al., 2013, Giedd et al., 1999), and increased parietal activity (Tapert et al., 2004b, Petit et al., 2014). Changes at a cellular level have also been noted, there is a very high rate of the production of axons and synapses in early adolescence, followed by a rapid reduction in late adolescence (Crews et al., 2007, Giedd et al., 1999, Andersen and Teicher, 2004, Andersen et al., 2000). Critical neural regions such as the hippocampus and the nucleus accumbens show extensive growth, pruning and remodelling during this period (Crews et al., 2007, Tarazi et al., 1998, Teicher et al., 1995, Boutros et al., 2015). Therefore it is logical to predict that any interruption or inhibition of this large-scale plasticity during adolescence may result in lasting damage (Crews et al., 2007, Vetreno and Crews, 2015). Therefore, it is possible that exposure to high concentrations of alcohol that are associated with binge drinking could lead to alterations of developmental plasticity, and may lead to lifelong detrimental effects (Skala and Walter, 2013).

It is the opinion of some groups that due to the similarities between adolescent binge drinkers and adult alcoholics, these conditions should be considered as the same condition, albeit in difference severities. This was termed the continuum hypothesis (Wagner and Anthony, 2002, McCarty et al., 2004, Enoch, 2006). Worryingly, there is a strong correlation between early onset of drinkers and the development of future alcoholism and other alcohol use disorders (Hingson et al., 2005, Miller et al., 2007, Bell et al., 2014, Lopez-Caneda et al., 2014a, Noel, 2014, Healey et al., 2014, Courtney and Polich, 2009, Barkley-Levenson and Crabbe, 2014, Thiele and Navarro, 2014, Viner and Taylor, 2007, Grant and Dawson, 1997, Maldonado-Devincci et al., 2010).
Multiple studies of human adolescents have shown that binge drinking can result in neurodegeneration in multiple brain regions (Crews et al., 2004). Such as reductions in; prefrontal volumes (De Bellis et al., 2005, Medina et al., 2008) and performance (Mota et al., 2013), the amount of adult neurotransmitter gene expression (Coleman et al., 2011), hippocampal volumes, that were directly correlated with the length of time they had been binge drinking (De Bellis et al., 2000, Medina et al., 2007, Nagel et al., 2005), cognitive performance derived from the frontal cortex and hippocampus (Tapert et al., 2004a, Schweinsburg et al., 2010, Squeglia et al., 2012, Squeglia et al., 2011). Even a single binge drinking episode could lead to neurodegeneration and cognitive impairment in humans (Obernier et al., 2002b), there is speculation as to whether this may underlie some of the cognitive dysfunction seen in binge drinkers (Vetreno and Crews, 2015, Vetreno et al., 2015, Liu and Crews, 2015, Boutros et al., 2015, Vetreno et al., 2014, Coleman et al., 2014). Although all of this speculation has come from a single group, with no one else yet having published any replications. Others have found significant decreases in cortical thickness in the right middle frontal gyrus in adolescent binge drinkers. They also reported unusual development of white matter in the right hemisphere precentral gyrus, lingual gyrus, middle temporal gyrus and anterior cingulate (Luciana et al., 2013). Others report, greater ventral striatal grey matter volumes (Howell et al., 2013), a lack of behavioural flexibility (Coleman et al., 2014), a reduction in axial diffusivity in the cerebellum, hippocampus and neocortex (Vetreno et al., 2015). Disturbingly, some adolescent drinkers show compromised white matter integrity – usually only seen in adult alcoholics – as early as mid-adolescence, even in those whom do not meet criteria for alcohol abuse or dependence (McQueeny et al., 2009). However, many of these studies (unless stated) did not control for differences in gender, which is known to be an important factor in human cognitive deficit performances in relation to binge drinking (Squeglia et al., 2012). An uneven balance of men or woman in any of these studies could then reflect unfairly on the other genders outcomes.

The neurodevelopmental pattern seen in humans during adolescence is also seen in other mammals, who even display increases in the same behaviours. Rodents, for instance increase; social interaction with their peers, levels of play, huddling and grooming (Fassino and Campbell, 1981, Ehardt and Bernstein, 1987). The effects of ethanol are also shared between humans and rodents, such as motor incoordination, increased social interaction and sedation are all characteristics associated with high, comparative levels of ethanol. Suggesting that the neuronal mechanisms underlying these effects is conserved between species (Davies et al., 2003a).
Both humans and rodent adolescents are visibly less affected by alcohol than adults when given the same doses (Spear, 2013). Moreover, adolescent rats self-administer up to three times more ethanol (in terms of kilogram per body weight) than adult rats (Doremus et al., 2005, Walker et al., 2008, Chin et al., 2010, Alaux-Cantin et al., 2013). Interestingly there is a difference in the kinetics of ethanol action between adolescent and adults. And lower serum ethanol concentrations have been shown in adolescent Sprague Dawley rats after i.p injection of ethanol when compared to adults (Little et al., 1996). However, this has been disputed in Wistar rats where no differences were found between adolescents and adults (Walker and Ehlers, 2009, Fleming et al., 2013). Also the neurons of adolescent rats seem to have a greater sensitive to ethanol neurotoxicity, and adolescent binge drinking resulted in frontal lobe degeneration (Crews et al., 2000, Vetreno et al., 2015, Liu and Crews, 2015, Coleman et al., 2014), and greater hippocampal impairments in adolescents than adults (White and Swartzwelder, 2004). Also ethanol treatments can lead to a loss of neurogenesis (Crews et al., 2006, Anderson et al., 2012). The role of neural progenitor cells in the dentate gyrus and their involvement in neurogenesis in the hippocampus is increasing in popularity (Kempermann et al., 2004, Imayoshi et al., 2008), and are suggested to have a role in learning and memory (Gould et al., 1999). As ethanol has a significant effect on neurogenesis this could play an important role in how ethanol affects memory (Briones and Woods, 2013). There is also evidence that adolescent ethanol treatments in rats and mice have shown increased mRNA expression of corticotropin release factor in the paraventricular nucleus of the hypothalamus (Przybycien-Szymanska et al., 2011), decreased neuropeptide Y in the hippocampus and increased substance P and neurokinin IR in the caudate (Slawecki et al., 2005, Lerma-Cabrera et al., 2013), reduced myelin density in the mPFC in (Vargas et al., 2014), increased impulsivity and attentional dysfunction in later life (Sanchez-Roige et al., 2014), and decreased activity in the adult PFC and enhanced activity in the NAc in response to further doses (Liu and Crews, 2015, Lacaille et al., 2015).

Despite the similarities between rodents and humans there is still difficulty in modelling human drinking alcohol consumption in animals. Humans often drink socially (Ernst et al., 2009, Spear, 2000), this social effect is difficult to implement in rodents. The rate at which ethanol is metabolised also differs between species. This is countered in some way by treating each species with different ethanol doses based on their metabolic rate, but this cannot negate this effect (Crabbe et al., 2011).
1.3.6 The use of animal models to study binge drinking

The need for an animal model to investigate binge drinking is essential, if investigation is to probe into the cellular neuro- and electro-physiology that may be being altered by binge drinking, then this cannot be performed ethically in humans. However, alcohol abuse and alcoholism are complex and no single animal can encapsulate all of the individual issues involved in all the behaviours (Crabbe et al., 2011, Rhodes et al., 2005). Therefore it is necessary to develop multiple models that each specialise in a particular feature of alcohol use (Thiele and Navarro, 2014).

One such fundamental aspect of binge drinking is the rapid escalation of BAC. Therefore, by performing this in animal models it is possible to investigate any effects this key feature of binge drinking has. Rodents have a wide range of alcohol preferences (Richter and Campbell, 1940), and are commonly used in the study of various types of alcohol use (acute, binge and alcoholism). Animal models that can be utilised to receive repeated high levels of ethanol either by free-choice or by investigator procedure make them a good model for studying ethanol binges and acute withdrawal (Bell et al., 2014). To study for the free-choice alcohol preference towards alcohol in particular breeds, tasks such as the two-bottle choice paradigm can be used. In this task, animals, typically mice and rats can choose between two bottles, one with an ethanol solution the other water. This type of experiment has discovered the alcohol preferences of many rodent models. Some breeds display alcohol-seeking behaviours such as; P-rats (Bell et al., 2011), HAD-1 rats (Clark et al., 2007), C57BL/6J mice (Yang et al., 2008, Kamdar et al., 2007, McClearn, 1959), and some alcohol-avoiding behaviours; DBA2/J mice (McClearn, 1959, Belknap et al., 1977, Fish et al., 2010) and NP-rats (Le and Kiianmaa, 1988). The C57BL/6J is a common mouse model for the investigation of alcohol use and misuse. It is genetically predisposed to consume high levels of ethanol, shown by studies of both self-administration and free drinking (Risinger et al., 1998, McClearn, 1959). Moreover, this strain consumes ethanol in levels equivalent to or greater than 2g/kg within 30 minutes (Finn et al., 2005, Tanchuck et al., 2011).

There are models that attempt to model binge drinking such models exist to reveal certain characteristics of binge drinking, each with their own strengths and weaknesses. Models like the ‘intermittent ethanol Injection’ model (IEI) (Pascual et al., 2009, Pascual et al., 2007), often referred to as ‘adolescent ethanol exposure’ (AIE) in this thesis due to its prevalence in assessing the effects of binge-like exposure in adolescents (Boutros
et al., 2014, Boutros et al., 2015, Sanchez-Roige et al., 2014, Alaux-Cantin et al., 2013, Lacaille et al., 2015). There is the ‘Drinking in the Dark’ model (DID) (Thiele and Navarro, 2014, Barkley-Levenson and Crabbe, 2014), and also the various Chronic Intermittent Ethanol (CIE) (Sabeti, Sabeti and Gruol, 2008, Cagetti et al., 2004) models. Other models have been developed to assess motivational attention towards alcohol, such as the free-drinking and two bottle choice paradigms (Griffin, 2014, Cacace et al., 2011). Other models aim to investigate acute alcohol – the equivalent of a single human drinking session (Moykkynen and Korpi, 2012, Dolganiuc and Szabo, 2009). There is a lot of research into alcoholism and therefore multiple models investigating chronic alcohol use, this is often performed by treating rodents daily with alcohol (Scholz and Mustard, 2013, Farris and Miles, 2012, Moonat et al., 2010).

One model where mice reliably and willingly drink to intoxication is the ‘drinking in the dark’ (DID) model (Thiele and Navarro, 2014, Barkley-Levenson and Crabbe, 2014). In this model, typically mice or rats are given access to a bottle containing a 10 – 30% ethanol solution for 4 hours, 3 hours after the ‘day lights’ are turned off. In these circumstances it is known that the mice will consume physiologically relevant concentrations of alcohol in a non-stressful environment (Rhodes et al., 2005, Mulligan et al., 2011). The DID model has a number of advantages, it uses natural ingestive drinking at night, and does not rely on water restriction to increase voluntary ethanol consumption or injection/gavage procedures (Ho and Chin, 1988, Sprow and Thiele, 2012). However, despite this it is impossible to control for the BAC in these animals. Moreover, free drinking procedures do not always result in the high level BACs associated with binge treatment (Matson and Grahame, 2013, Barkley-Levenson and Crabbe, 2014). Therefore in studies where the controlling the BAC is important, the use of researcher-administrated ethanol is preferred (Dolganiuc and Szabo, 2009).

Another model that has been used to closer study the BAC during drinking are the variations of CIE. This model involves exposing rodents to ethanol every other day, via a researcher administrated procedure such as a vapour inhalation chamber, injection or gavage (Moonat et al., 2010). The degree of control of ethanol exposure varies between techniques. Vapour inhalation, for instance involves cycles of 14h exposure to ethanol – 10h ethanol free for a period of 12 – 14 days. This technique typically results in relevant BAC’s to a human binge cycle (Roberto et al., 2002, Rogers et al., 1979). Moreover, CIE vapour inhalation allows rodents to experience multiple cycles of ethanol intoxication and drug-free. Variations of concentration and duration can be produce ethanol dependence
in adolescent and adult rats (Sabeti and Gruol, 2008, O'Dell et al., 2004, Slawecki et al., 2004).

The model of binge drinking that is used in this thesis was originally developed by Pascual. The intention was that each young rodent would have received 8 ethanol doses simulating an intermittent drinking pattern that is characteristic of adolescent and young adults (White et al., 2006, Pascual et al., 2007). Since the original use of the formerly titled IEI, now AIE 2-day-on 2-day-off procedure, it has gained support from multiple labs and used in multiple models. For instance it is now used Wistar rats (Boutros et al., 2015, Boutros et al., 2014, Vetreno et al., 2015, Liu and Crews, 2015, Vetreno et al., 2014, Vetreno and Crews, 2015), Sprague Dawley rats (Lerma-Cabrera et al., 2013, Forbes et al., 2013), and C57Bl/6J mice (Sanchez-Roige et al., 2014, Rodriguez-Arias et al., 2011). This model results in highly reproducible and relevant BAC's in a binge-like pattern (Pascual et al., 2007, Alaux-Cantin et al., 2013), and benefits from precision in controlling the administration quantity and duration. However, a disadvantage of increasing the control of BAC's is a fall in the ‘realism’ of alcohol administration. As it has been shown that administration of alcohol i.p. differs from self-administration of the same amount of alcohol (Jacobs et al., 2003).

One of the real benefits of using the AIE model is that the time course and concentrations after a single i.p. injection has been thoroughly calculated. After a single i.p. injection the BEC rises and peaks at $210 \pm 11$ mg/dl at 30 minutes post-injection. After this the ethanol concentration gradually reduces until around 540 minutes after the injection (Pascual et al., 2007, Lacaille et al., 2015). A study showed that a 1.75g/kg i.p. injection resulted in $\sim 200 – 220$ mg/dl at around 30 minutes in C57BL/6J mice, and this remained similar with subsequent injections (Linsenbardt et al., 2009). Although this isn’t the exactly the same dose as used in the AIE treatment in the thesis, it is very similar. Therefore it could be expected that the AIE used here could achieve slightly higher BAC levels than 220 mg/dl. Interestingly in humans these higher BAC’s $\sim 20 – 40$ mm which is $\sim 100 – 200$mg/dl, result in the altered cognitive processes, towards the top end or higher and some individuals will develop memory anterograde amnesia or blackouts (White, 2003, Zorumski et al., 2014).
1.4 Aims of the thesis

This thesis aims to investigate the effects that high concentrations of ethanol have on learning and memory. By using both acute, single injections of ethanol and ethanol treatments that were designed to model elements of human binge drinking. Where possible it was the preference to investigate this during late adolescence.

To do this, two different animal models were used. A frequently used model used to investigate alcohol use, the C57BL/6J mouse (Talani et al., 2013, Cox et al., 2013, Rice et al., 2012, Lowery-Gionta et al., 2012, Talani et al., 2011, Melon and Boehm, 2011, Melendez, 2011, Kiselycznyk and Holmes, 2011, Holstein et al., 2011, Hwa et al., 2011). I also developed a new model for the effects of alcohol on memory and single neuronal activity, the snail *Lymnaea stagnalis* which has, along with related molluscs, been extensively used to investigate the evolutionarily conserved cellular and molecular mechanisms of learning and memory (Kemenes, 2013, Rosenegger and Lukowiak, 2010, Marra et al., 2010, Kennedy et al., 2010, Cheung et al., 2006, Kojima et al., 1997, Kemenes et al., 1997, Rubakhin et al., 1996).

Chapter 2 covers the fundamental methods used in many of the chapters. Chapter 3, explores the effects that ethanol treatments, performed during late adolescence, have on hippocampal-dependent spatial memory tasks. Chapter 4 expands on this by investigating what these late adolescent ethanol treatments are having on hippocampal LTP directly, by using hippocampal electrophysiology and measuring the field potentials of the pyramidal cells in the CA1 whilst stimulating the CA3 region. Chapter 5, builds on the investigation of memory tasks in chapter 3 by performing ethanol treatments whilst training on a lever pressing task. Chapter 6, introduces the snail model *Lymnaea* and investigates the how ethanol affects learning and memory using a single-trial classical conditioning paradigm. This model has well defined temporal molecular mechanisms, allowing for ethanol to be investigated on acquisition, consolidation and retrieval via a single well-timed injection. Chapter 7, investigates the effect of ethanol on the *Lymnaea* CGC, a large modulatory cell with known involvement in plasticity and memory within the single-trial classical conditioning paradigm. This completes the use of both behavioural and electrophysiological investigations in two separate animal models. Chapter 8, discusses the thesis as a whole and compares and contrasts between the studies presented in the previous chapters, and ending in a final conclusion.
Chapter 2 – Methods

The following two sections provide the base methods used in the experiments reported in this thesis. Where altered or additional methodology was used it will be stated prior to the relevant results.

2.1 Methodologies utilizing the C57BL/6J mouse

2.1.1 C57BL/6J mice

All experiments in chapters 3, 4 and 5 used male C57BL/6J mice (Charles Rivers, UK). The age of the mice was controlled for. They were either used during the late adolescent period post-natal days (PND) 45 – 60, or at the start of young adulthood PND 60. All mice were pair housed (unless specifically stated) and kept in standard housing conditions (NKP cages, M2, overall size: 33 x 15 x 13cm; internal size: 300cm³ x 13cm). Rooms were temperature (21 ± 2°C), and humidity (50 ± 5%) controlled, and lit on a 12-hour light/dark cycle with lights on at 7:00. Animals were fed rodent chow (Special diet services – 801960) and had access to water ad libitum throughout the experiment. All mice were handled daily to reduce anxiety. Experiments were approved by the institutional ethics committee and were performed under United Kingdom legislation on animal experimentation [Animals (Scientific Procedures) Act, 1986].

2.1.2 Apparatus

2.1.2.1 Video recording and analysis

All sessions in the MWM, locomotion and the object recognition task were recorded using a Sony hyper HAD camera mounted above the pool/arena (under the cylinders in the locomotion task) and connected to a computer for recording and later analysis. Analysis in the MWM and object recognition task was performed by the Ethovision (Noldus) software. The locomotion task was analysed by a custom MatLab program.
2.1.2.2 Morris water maze

The Morris Water Maze consisted of a circular pool with an internal diameter of 83cm filled with water (22-24°C). A clear escape platform (12cm height, 23.5 cm circumference) was submerged at a level that could not be detected by the animal whilst swimming but would allow the animal's body to be out of the water when standing on the platform. Extra-maze cues were kept in a constant position around the pool, such as tables and chairs. Additionally, there were four distinct A4 printed geometric designs placed around the maze.

The pool was divided into quadrants and compass points were arbitrarily labelled North, East, South and West (N, E, S and W) for ease of navigating around the pool. The platform was placed 12 cm from the edge of the pool and at a midpoint between 2 compass points (NE, SE, SW and NW).

2.1.2.3 Operant recording chambers

Two highly similar, but different operant chambers were used between phases 1 and 2.

8 operant chambers (22.5 × 18 × 13cm; Med Associates, Georgia, VT, USA) were used in phase one. Each was fitted inside a sound-attenuating wooden box, with a ventilation fan. Each chamber had a food magazine (W = 2.5cm, H = 2cm) in the bottom centre of the right wall, which was connected to a dispenser that released 2mg sucrose pellets upon a correct lever press (Sandown Scientific, Middlesex, UK). A house light was fixed in the top-centre of the left wall. The floor comprised of a metal grid which allows for sawdust and faeces to fall through. The chambers were controlled by Med-PC (version 5) and the tasks programmed in Medstate notation.

Phase two used different operant chambers to phase one, although aesthetically different they were structurally very similar, all levers and magazines were in the same positions and of the same size, the floor and overall size of the boxes were matched. There were two noteworthy differences. First, the levers in phase two required less force to give a response to the food reward. Second, the food reward was changed from sugar pellets to 17.7µl of strawberry milkshake (Nesquick strawberry milkshake, 50g of milkshake powder mixed with 150ml Co-op UHT 0% fat milk). This change was to
observe whether there was some conditioned taste aversion towards the sugar pellet food-reward.

2.1.3 Experimental procedures

2.1.3.1 Adolescent intermittent ethanol AIE treatment

Several experiments perform AIE treatment beginning on PND 45. The AIE group are injected intraperitoneal i.p with ethanol (2g/kg, 20%v/v in physiological saline, i.p.) on a 2-day-on 2-day-off protocol for a total of 14 days (8 ethanol injections). The control AIE group received equivalent saline injections following the same temporal injection protocol. In a typical experiment mice were given 7 days rest after the final injection before the start of experimentation. This rest period allows for the acute withdrawal from the ethanol treatment to dissipate (Pascual et al., 2007, Sanchez-Roige et al., 2014), as the interest is what occurs during a ‘binge-like’ treatment and not the acute effects of withdrawal itself.

2.1.3.2 Hippocampal slice procedure

Dissection

Animals were killed by cervical dislocation and the brain removed. Dissected brains were placed into chilled (4°C) oxygenated aCSF (artificial cerebral spinal fluid), and transported on ice to the tissue slicer in the recording room. Hippocampi were removed from the brain and sliced into 250μm thick slices, and were left to recover in room temperature oxygenated aCSF for between 30-60 minutes.

Recording

Before recording, slices were placed into the recording chamber where they were constantly perfused with oxygenated aCSF at temperature 28±2°C. A stimulating electrode made from two pieces of tungsten wire separated by capillary tubes, which were melted to a fine point, was placed on the Schaffer collateral on the CA2-CA1 border. A recording electrode, a 3-5 MΩ glass electrode pulled by a machine was placed just
outside of the pyramidal cell body layer of the CA3, towards the Schaffer collateral. Test pulses (100µs) occurred every 30s at intensities between 4-30V to evoke a fEPSP. The optimum stimulus intensity is the midpoint between the voltages that result in the minimum and maximum fEPSP. To generate this, stimulus intensities were slowly raised (typically between 6 – 30mv) until an input/output curve displayed the minimal and maximal response. The voltage at the midpoint of the I/O curve was chosen as the stimulus intensity for recording. LTP was induced by a single HFS of 1s at 100Hz resulting in 100 pulses at standard stimulus intensity (see Fig 1.3).

2.2 Methodologies utilizing *Lymnaea stagnalis*

2.2.1 Animals

Common pond snails (*Lymnaea stagnalis*) aged 3-4 months were collected from the in house breeding facility (originally obtained from the Free University, Amsterdam). This age corresponds to the start of adulthood, which is assumed to correspond late adolescence in mammals. Although it must be explicitly stated that the complexity of mammalian adolescent development cannot be accurately modelled in *Lymnaea*, it was of the view that the learning behaviour and nervous system of snails of this age can be used to study the most fundamental effects of ethanol. All snails were kept on a 12Hr light/dark cycle at ~20°C and fed on a combination of fresh lettuce, 3 times a week, and fish food (Tetra-Phyll, TETRA Werke) twice a week. Petri dishes were used as arenas for the snails to be conditioned and tested. All injections were made using 30G x 1/2” BD microlance needle tips and 1ml BD Plastipak syringes.

2.2.2 Solutions

Ethanol was obtained from Sigma Aldritch, UK. Solutions were prepared by diluting a 95% stock solution with a snail specific saline solution (v/v) (50mM NaCl, 1.6mM KCl, 2mM MgCl\(_2\).6H\(_2\)O, 3.5mM CaCl\(_2\).2H\(_2\)O, 10mM HEPES). A 0.67% sucrose solution (in water) made using analytical sucrose (Fisher, UK) was used as the un-conditional stimulus (US). 0.004% amyl acetate (Sigma Aldritch, UK) diluted from concentrate with water was used as the NS/CS.
Chapter 3 – The effect of AIE treatment on spatial memory in C57BL/6J mice

Binge drinking is highly prevalent among adolescents, studies have shown that this can affect various forms of memory, including spatial memory (Weissenborn and Duka, 2003, Townshend and Duka, 2005, Tapert et al., 2004b, Spear, 2015). Spatial memory is relatively easy to test for in rodents, and therefore offers an opportunity to investigate the effects that binge-like treatments have on spatial memory. This chapter will provide and exploration of the effects that AIE (adolescent intermittent ethanol) – a model for adolescent binge drinking – has on the spatial and non-spatial memory performance of C57BL/6J mice.

The chapter begins with a discussion of spatial memory and how spatial memory can be tested in rodents. This is followed by a review of the ways in which acute, repeated acute, AIE and CIE ethanol treatments affect spatial and non-spatial memory with a particular focus on differences between adulthood and adolescence. Four experiments are then described, the first investigating the effects that AIE treatment has on later life performance in the MWM and the extent to which learning depends on spatial or non-spatial strategies. The second experiment, investigates the effects that AIE treatment has on MWM performance when both the MWM and AIE treatment are performed during the same period. The third experiment investigates the effects that AIE treatment has on later life performance of both a spatial and non-spatial version of the object recognition task. Finally, the effects of AIE treatment on a standard assay of locomotor activity are described.

3.1 Introduction

3.1.1 Spatial memory and navigation cells

As described in the general introduction, binge drinking, or ethanol treatments attempting to model the physiological effects of binge drinking, can lead to deficits in many types of memory. One particularly testable type of memory that is known to be affected by binge drinking is spatial memory (Weissenborn and Duka, 2003, Townshend and Duka, 2005, Tapert et al., 2004b).
Spatial memory has been previously discussed in the general introduction. To briefly recap, spatial memory contains all the information that defines a particular environment and the movement and actions taken when in or getting to that place. It profiles the surrounding external 3D space to create mental maps (O'Keefe and Dostrovsky, 1971), which can later be recalled and used to navigate to the same location (Vorhees and Williams, 2014). Non-spatial strategies to achieve the same goal are independent of the surroundings and are internal to the organism, such as a list of memorized movements to reach a destination without using other sensory stimuli (Garcia-Moreno and Cimadevilla, 2012, Vorhees and Williams, 2014).

Spatial memory is reliant on a type of hippocampal cells called place cells, which were originally found in the hippocampus of rats (O'Keefe and Dostrovsky, 1971, O'Keefe, 2007, Stark, 2007). By recording the complex-spike cells of the CA1, O'Keefe et al began to notice that certain cells fired when the subject was in a specific place, these cells became known as place cells. Place cells are involved in spatial memory are responsible to maintain a map of the environment including individual objects and the direction and distance between them (O'Keefe, 1976, O'Keefe and Nadel, 1978, O'Keefe, 2007).

Navigation is not solely performed by place cells. It was later hypothesised that place cells receive input from HD cells for the directional information (O'Keefe, 1991), and that certain place cells were activated depending on far away an object was, as input from HD cells (McNaughton et al., 1996). Head-direction (HD) cells, which were first discovered in the dorsal pre-subiculum (Taube et al., 1990, Taube, 1998, Ranch, 1984). Although they have also been shown in the anterior and lateral thalamic nucleus (Taube, 1995, Mizumori and Williams, 1993), lateral mammillary nucleus (Stackman and Taube, 1998), retrosplenial cortex (Chen et al., 1994, Cho and Sharp, 2001), and striatum (Wiener, 1993). HD cells fire in various headings relative to the environment, and are individually sensitive to particular directions (O'Keefe, 2007). Supposedly, HD cells are continually updated as the subject moved through an environment using feedback from the vestibular and proprioceptive systems (O'Keefe, 2007). Supporting this is the finding that lesions of the vestibular complex causes the HD cells to alter their specific locations (Stackman and Taube, 1997).

There is another component to the functioning of spatial memory in the hippocampal formation, and this is grid cells. Grid cells have been found in the layers 2/3 of the entorhinal cortex where they project towards the hippocampus. These cells are responsible for processing a grid-like map of every environment that the subject
encounters. They provide the place cells with necessary distance and directional information (O'Keefe, 2007)

More recent work has shown the importance of the NMDA receptor in place cell functioning, and therefore spatial memory (Nakazawa et al., 2004, Bannerman, 2009). The NMDA NR1 subunit, when selectively knocked-out in the CA1 pyramidal cells causes disruption of LTP and spatial memory (McHugh et al., 1996), adding support for a link between hippocampal LTP and spatial memory. Mutants of the GluN2 NMDA receptor showed impaired spatial memory indicating an involvement of the GluN2 NMDA receptor in spatial memory (Bannerman, 2009). Additionally, the same result was found when using a mutated form of CaMKII, which is downstream of NMDA receptor activation and necessary for hippocampal LTP (Rotenberg et al., 1996, Byth, 2014, Mayford et al., 1996). Suggesting that spatial memory is dependent on the NMDA receptor pathway and not just NMDA activation (Tsien et al., 1996b, O'Keefe, 2007). Interestingly these experiments found fewer place cells in both of those knockout animals, and that the place fields themselves were disrupted (Cho et al., 1998, O'Keefe, 2007). NMDA receptors appear to be involved in long-term memory consolidation and stability of hippocampal place fields (Kentros et al., 1998, O'Keefe, 2007). Since, the NMDA channel blocker CPP had no inhibitory effect on existing place fields or the mapping of new novel place fields, or even place field STM. However, it did effect performance of a previous day. It is not fully known however that all of these changes occur in the hippocampus itself and not related structures (O'Keefe, 2007).

### 3.1.2 Why and how to asses spatial memory

Unlike other forms of explicit memory, spatial memory is easy to assess in rodents. There is a wide variety of spatial memory tasks that have been developed over the past 50 years. Mazes such as the T-maze, radial arm maze, Barnes maze and the Morris water maze (MWM), although some, if not all, of these tasks can also be solved using non-spatial strategies (Sharma et al., 2010). As the effects of binge drinking and AIE type treatments may have relatively minor effects on later life neural functioning, it was felt that a ‘difficult’ maze should be used.

The T-maze involves the subject making a simple left or right choice, similar to a ‘T junction’, hence its name. This is a relatively simple maze and although it was been shown to involve the hippocampus (Sharma et al., 2010), other learning strategies can
be used that rely on the caudate nucleus (Packard et al., 1989, Packard and McGaugh, 1996). A more complex version of the T-maze is the radial arm maze. This maze gives the subject several (6 – 10) direction choices and benefits from being low-stress (Hodges, 1996). However, the task can be completed by simply going from one arm to the next, negating the use of spatial cues and therefore not testing spatial memory (Sharma et al., 2010), though in practice rodents do not usually use such a strategy. The Barnes maze is a large diameter disc with holes in, where one hole is the correct hole, which contains a dark box (Sharma et al., 2010). This is similar to the Morris water maze, but since the Barnes maze is a dry maze there is little reinforcement to locate the correct hole other than the desire to be in the dark box, this sometimes makes learning the task difficult and long. It is also necessary to include controls for odour cues that may influence performance on successive trials. The MWM benefits from being a relatively ‘difficult’ task in which the rodents use a variety of spatial cues to locate the escape platform (Sharma et al., 2010, Morris et al., 1982). Therefore it was decided that the MWM would be one of the best techniques to use to evaluate the effects of AIE treatment on spatial memory.

### 3.1.2.1 The Morris water maze

In the MWM animals are placed into a pool of water and are required to locate and climb onto a hidden escape platform to successfully complete the task. Completion of the MWM requires the acquisition of a cognitive map, detailing the pool and the surrounding external spatial cues (Morris et al., 1982, Vorhees and Williams, 2014). After some training the animals will learn to use internal and external cues to locate the hidden platform (Berry and Matthews, 2004, Santin et al., 2000, Morris, 1984). Some of the benefits from using the MWM task are the lack of alternative cues (such as olfactory cues that could be left on a dry maze), there is no need for appetitive reinforcement which might require a particular degree of motivation to be present (such as food deprivation when a food reward is used), and no negative reinforcement (such as electric foot shock) (Sharma et al., 2010, Vorhees and Williams, 2014).

Although the MWM task was originally designed for use in rats (Morris, 1984, Brandeis et al., 1989), other rodents have been successfully used, including the mouse. The mouse shows some behavioural differences to the rat during swimming tasks such as increased thigmotaxis (swimming around the walls) and floating behaviours. They have a slightly slower swim speed than rats, and take longer to complete the task (D’Hooge and De Deyn, 2001). However, this is not thought to be due to a poorer spatial memory,
and there is reason to believe that the difference could be down to natural swimming strength (Whishaw and Tomie, 1996). Studies have supported the idea that the C57BL/6J mouse strain is fully capable of acquiring the MWM task, and that they are also successful in completing spatial memory dependent probe trials, a test involving the removal of the escape platform (Stavnezer et al., 2002, Wright et al., 2004). If the animal spends the majority of the trial in the correct quadrant, near where the platforms previous location, then the animal has acquired the task well (Berry and Matthews, 2004, Whishaw and Tomie, 1996, Wright et al., 2004, Cho et al., 1999). The C57BL/6J mouse strain has also been shown to either out-perform or match the highest performing mouse strains when acquiring the MWM (Wright et al., 2004). And again, in probe trials their performances are comparably high, and they spend the majority of the time in the correct quadrant. Together these results suggest that C57BL/6J mice have a stronger spatial learning and memory capabilities than many other mouse strains (Wright et al., 2004).

It was from experiments such as the MWM that showed the crucial involvement of the hippocampus, and that the hippocampus is crucial for correct spatial memory function (Morris et al., 1982, Morris, 1984, Langston et al., 2010). Studies in rats have shown that when the hippocampus and its extrinsic fibre connections are lesioned the result is a profound and long-lasting impairment in MWM performance (Morris, 1984, Morris et al., 1982). The behavioural specificity of these lesions have been demonstrated as hippocampal lesions in male Lister-hooded rats have been shown to impair spatial memory in hidden (spatial memory dependent) but not visible (non-spatial memory dependent) platform mazes (D’Hooge and De Deyn, 2001, Pearce et al., 1998). This finding was later replicated using C57BL/6J mice, where ibotenate lesions of the hippocampus resulted in an inability to accurately learn the platform position in the MWM, and during the probe a failure to choose the correct quadrant. However, the same ibotenate lesioned mice, when trained on a non-spatial version of the task, performed equally well as to the mice who received sham lesions (Cho et al., 1999). Indeed, some studies have shown that animals with hippocampal lesions, and therefore disrupted spatial memory, are able to acquire non-spatial tasks at a faster rate than controls (Packard et al., 1989). Studies employing the inactivation of the hippocampus using a AMPA/Kainate glutamate antagonist, that allows the experimenter to temporarily, and without lasting damage, “turn off” the hippocampus, have demonstrated that the hippocampus is necessary for both encoding and retrieval of spatial memory in the MWM task (Riedel et al., 1999).
3.1.2.2 The spatial and novel object recognition task

Another memory task that allows for the testing of the effects of AIE treatment on spatial and non-spatial memory is the object recognition task. This task uses the natural tendencies of rodents to explore their surrounding environments (Berlyne, D.E, 1950). This exploratory behaviour is particularly useful because of their tendencies to explore novel over familiar objects (Vorhees and Williams, 2014). Similar to the MWM, the OR task requires minimal external reinforcement. Typically, there are two or three objects in the centre of an open field, after a number of trials the rodent will have learnt the relative spatial positioning of the objects. At a later trial, one of the objects can be relocated to a different position and the amount of time that the animal spends exploring the relocated object would be a measure of their ability to detect spatial changes, creating a spatial memory test. Also within the same experiment a familiar object can be replaced by a novel object, producing a test of non-spatial memory (Ennaceur and Delacour, 1988).

Like in the MWM, the hippocampus has also been shown to be critical for the completion of the OR task (Mumby et al., 1996, Phillips et al., 1988). Lesion studies have shown that hippocampal-entorhinal connections were more important for the identification of a novel geometric arrangement of objects (spatial test) than for place navigation (Parron et al., 2006). Whereas others have suggested a similar role for the dorsal dentate gyrus and hippocampus, where it is hypothesised that they are more involved in the processing spatial feature and patterns (Hunsaker et al., 2008, Kesner et al., 2015). With lesions of the dentate gyrus resulting in an impairment of novel object recognition, where lesions of the CA3 have no effect (Okada and Okaichi, 2009). However, there is a debate on the involvement of the hippocampus in the OR task (Barker and Warburton, 2011).

The entorhinal and perirhinal regions have received particular attention and have been implicated in the memory of spatial relocation of objects within the object recognition task. Lesions of the rat entorhinal cortex resulted in poorer performance in the spatial relocation of objects in the object recognition task (Parron and Save, 2004). Also, lesions in the perirhinal cortex also severely disrupt novel object recognition, a non-spatial memory test, and the recognition of object spatial changes (Bartko et al., 2007, Norman and Eacott, 2004, Mumby and Pinel, 1994, Ennaceur et al., 1996, Bussey et al., 1999).
3.1.3 The effect of ethanol on spatial memory

As has been mentioned the consumption or treatment of ethanol in a binge pattern has been shown to impair hippocampal function (Vetreno and Crews, 2015, White et al., 2000b). Given that the hippocampus proper, along with the hippocampal formation are crucial in spatial memory and successful completion of MWM and object recognition tasks. It is logical to hypothesise that AIE treatment and/or acute ethanol could impair spatial memory and therefore performance in spatial memory tasks, such as the MWM or object recognition task. There have been numerous investigations into the effects ethanol has on spatial memory. Therefore a concise review of the relevant literature is essential.

3.1.3.1 The effects of acute ethanol on spatial and non-spatial memory in adolescents and adults

To test for the effect of acute ethanol on spatial memory, ethanol naïve rats were trained on either a spatial reference or non-spatial reference form of the radial arm maze. Testing was performed after injections of either ethanol (0.75, 1.5 or 2.25g/kg) or saline. The results indicate that acute ethanol impaired spatial reference memory in a dose-dependent manner, with no effect on non-spatial reference memory (Matthews and Silvers, 2004). Similarly when acute ethanol (1, 1.5 and 2g/kg) injection was investigated using the same experimental design, it temporarily impaired spatial working memory in a dose-dependent manner (Hoffmann and Matthews, 2001). Moreover, it was found that acute ethanol produced a dose-dependent shift in preference for non-spatial dependent choices over spatial memory dependent choices when compared with saline treated animals (Matthews et al., 1999). Even a dose as low as 1.25g/kg has been shown to impair spatial working memory in adult Long Evans rats when injected 15 minutes before testing in an 8 - arm radial arm maze (Gibson, 1985). Whereas similar doses (0.5, 0.75 and 1g/kg) in the MWM, a more cognitively demanding experiment, showed no impairment at any concentration in either adolescents or adults (Novier et al., 2012). However if the dose is increased to 1.75g/kg then a spatial memory impairment, but not non-spatial memory in the MWM is seen in C57BL/6J mice. However, when treated with a higher dose (2.25 g/kg), both spatial and non-spatial memory were impaired (Berry and Matthews, 2004). This same effect has also been reported in adult male Long Evans rats (White et al., 1998, Matthews et al., 2002), and adult male Wistar rats (Shimizu et al., 1998).
Many of these studies have been performed in the same lab. The account they give for ethanol’s action of spatial memory does not rule out the possibility that acute ethanol is affecting ability or motivation to complete these tasks. In fact, in the one example given above where ethanol did not apparently impair spatial memory there was negative reinforcement (MWM). These results could be explained by ethanol injection reducing the motivation to complete the task, but when there is another motivational aspect such a negative reinforcement, this drives the subject to complete to task. Nonetheless, injection of acute ethanol have still shown to impair performance in spatial memory in tasks such as the radial arm maze (Matthews and Silvers, 2004, Gibson, 1985).

Acute ethanol (2.4 g/kg) has also been shown to inhibit preferential exploration of novel vs. familiar objects (non-spatial) in the OR task (Ryabinin et al., 2002) without having a noticeable effect on locomotor activity. However, the experiment injected 2 minutes prior to training and as such there would have been a stimulatory early period of the experiment where the BAC would rapidly rise. Interestingly if ethanol was injected post-trial, performance in a novel object recognition task was increased (Brooks et al., 2002).

One example of how acute ethanol is different in adolescents than adults is that when adolescent female rats were treated with 2g/kg ethanol before training in the MWM, they found an impairment in spatial learning without affecting locomotor activity (Sircar et al., 2009). However, although adult female rats did show an impairment of acquisition like adolescents, they were not as equally as affected in the probe trials. Though their general acquisition of the task was poorer and this could be reflected in the greater loss in performance seen in adolescent female rats (Sircar et al., 2009). There is also suggestion that adolescent C57Bl/6J mice are more sensitive to the locomotor stimulant, anxiolytic and ataxic effects of ethanol when compared to adult mice (Hefner and Holmes, 2007). Interestingly though, the sedative effects of ethanol were less pronounced in adolescent mice than adults (Hefner and Holmes, 2007). Other examples show increased sensitivity in low doses of ethanol that do not effect adult. Where 1g/kg ethanol administered immediately before training, inhibited exploration of the novel object in adolescents, but not in adult C57BL/6J mice (Spanos et al., 2012).

To summarise, acute ethanol impairs spatial memory in a dose-dependent manner, and appears to have a lesser effect on non-spatial memory, with adolescent rodents suffering greater impairments than adults. Moreover, acute ethanol is not having any impact on
later performance when not intoxicated. Acute ethanol also impairs performance of a non-spatial test in the object recognition task. However, non-specific effects have not always been ruled out in these experiments.

3.1.3.2 The effects of binge-like ethanol treatments on adolescents and adults

Analysing the results of ethanol treatments becomes more complicated when ethanol is administered more than once, and it is still relatively unclear when a treatment becomes a ‘binge treatment’. For instance the following examples use repeated ethanol injections, most of these treatments do not last for longer than 5-7 days and often do not have days of drug-free days, something which is seeing increasing attention in the field (Bekman et al., 2013, Ripley and Stephens, 2011, Duka et al., 2004, Stephens et al., 2005). The differential effects seen between acute, repeated acute, binge (AIE, CIE, DID) and chronic treatments, adds to the complication of studying ‘binge drinking’ in rodent models. The effects of acute ethanol, although interesting, have limited reference when considering typical human drinking patterns. More recently the attention of alcohol researchers has moved towards the differential effects that ethanol use has on adolescents/young adults, and adults. With the prevalence of binge drinking among adolescents and young adults increasing (Petit et al., 2014, Lopez-Caneda et al., 2014b, Gross et al., 2014, Stickley et al., 2013), it is important to understand how ethanol is effecting their brains and if it could interfere with their development. A lot of focus has been directed towards modelling and understanding various treatments that in some way reflect binge drinking. Therefore allowing the study of certain neural regions and their corresponding transmission pathways and how these regions differ between adolescence than in adulthood.

When adolescent male Sprague Dawley rats were treated with 2g/kg 30 minutes before training in a MWM for 5 consecutive days. The ethanol treatment caused disruption of performance, the rats displayed increased thigmotaxis and even showed poorer performance up to 30 days later (Sircar and Sircar, 2005). It’s clear however, that this is a repeated acute ethanol procedure and ethanol is impairing the spatial memory during acquisition, possibly by effecting encoding of the memory. Other studies have shown contrasting results. One such study found that repeated ethanol treatment during adolescence increased susceptibility to the memory-impairing effects of ethanol in later life (White et al., 2000a). Another performed repeated acute ethanol injections 2g/kg in adolescence and adult Sprague Dawley rats 30 minutes before training in a MWM. Here ethanol treatment significantly impaired adolescent spatial memory acquisition of the
MWM, but did not impair adults (Markwiese et al., 1998). Moreover non-spatial was unaffected in either group.

In a study of the effects of adult binge-like ethanol treatments on performance in the MWM, Sprague Dawley rats that underwent a 2 week binge-like treatment, did not show impairments in performance on the MWM after being administered 1.5g/kg ethanol, 30 minutes before training (Boulouard et al., 2002). A binge model using adult male Wistar rats administered ethanol by gavage every 8 h for 4 days, resulting in doses of 9 to 15g/kg of ethanol per day. This led to impaired spatial and non-spatial memory in an object recognition task when tested one week after cessation of the binge-like treatment (Cippitelli et al., 2010). The same experiment tested another group of animals 10 weeks after cessation of the binge-like treatment, this group showed an impairment in spatial but not non-spatial memory in an object recognition task (Cippitelli et al., 2010). It is difficult to compare bolus injection treatments with prolonged gavage treatments though, the uptake kinetics and metabolism of ethanol will be remarkably different between the two administrative techniques. Moreover, perhaps an 8 hour treatment may better reflect chronic ethanol consumption than modelling binge drinking.

There has been very limited studies on the effects of AIE treatments on spatial memory. However, one study investigated AIE in early adolescent male Sprague Dawley rats, they were given repeated ethanol injections 5g/kg (i.p.), in a one-day on, one-day off pattern and were trained in the MWM on drug free days. After 4 weeks the AIE treatment produced no deficits in MWM acquisition or probe trials when tested after a break period (Schulteis et al., 2008). Using a very similar AIE treatment, but where the animals were given a break before training in a MWM, also suggests that AIE treatment had no effect on spatial learning in adulthood, however it was suggested that it did promote behavioural inefficiency (Acheson et al., 2013).

A study of AIE on the MWM found that AIE treatment produced no difference in acquisition of the MWM but did result in a short-term tolerance to the ethanol-impairing effects of spatial memory that was not evident 12 days after training finished (Silvers et al., 2006, Silvers et al., 2003b). Similarly, a study on the effects of AIE treatment on male adolescent Sprague Dawley rats showed altered hippocampal function that also developed a tolerance to ethanol-induced inhibition of spatial memory (Tokunaga et al., 2006). However, the opposite has been found in the same adolescent rat species. Where AIE treatments have potentiated later-life ethanol-induced impairments (White et al.,
Together these may suggest that AIE treatments are able to produce some long-term behavioural effects, although much more study is needed to understand and better model the human condition.

To summarise, AIE-like treatments are showing signs of behavioural modification of spatial memory in rodent models without having any profound effect of spatial memory, as has been shown in humans. Stronger treatments have certainly been shown to impair spatial memory, though there particular relevance to the human phenomena of binge drinking is questionable as it could be likely that these rodent models may have developed a dependence.

### 3.1.4 Aims and hypotheses

It is hypothesised that AIE treatments may share the spatial memory impairment that is seen in adolescent and young adult, human binge drinkers (Weissenborn and Duka, 2003, Townshend and Duka, 2005, Tapert et al., 2004b). By utilising well-known hippocampal-dependent spatial memory tasks such as the MWM and spatial and novel object recognition task, it is possible to test the effects of AIE treatments on spatial memory. However, human binge drinkers rarely, if ever, binge for an extended period, rest, then attempt to learn something. Therefore, it is important to model for this by performing AIE treatments either whilst intoxicated/drug-free during the training/testing period.

Therefore the overarching aim of this series of experiments is to expand the current knowledge of the effects of AIE treatments on adulthood spatial, and non-spatial memory.

The specific aims are:

1. To investigate the effects that AIE treatment has on later life hippocampal dependent spatial memory.
2. To investigate that impact that AIE treatment may have on the ability to perform spatial memory tasks while the AIE treatment is still ongoing. These experiments are designed so as to examine the effects of being tested when the animals are either intoxicated or drug-free.
3.2 Methods

3.2.1 General MWM procedure and analysis

Habituation: Training in the MWM started by exposure to a single habituation trial where the escape platform was placed in the centre of the pool to avoid bias in later sessions. The mice were placed on the central platform for 30s, followed by a 30s swim and 3 assisted climbs onto the central platform. Training: On each training trial, mice were placed into the pool facing the external wall, and had 60s to locate and climb onto the escape platform. Animals failing to complete the task received a score of 60s and were placed onto the platform. All animals remained on the platform for 10s before being removed from the pool. There was an ITI of 60s before the next trial began. Once the mice had finished that days experiments they were placed into a warming holding cell to dry. There were a total of 8 trial days. Probe trials: Probe trials were performed to assess a particular hypothesis. In the investigation of learning strategy the probe trials investigated whether the mice performed better using spatial or non-spatial memory after AIE treatment. In the investigation of the effects of AIE on acquisition of the MWM, the probe trials tested how each group were affected by a saline or ethanol injection prior to testing. Common to all probe trials was that probe trial 1 was conducted 48 hours after the final training session, and probe trial 2, 24 hours after the probe trial 1.

Acquisition was measured in all MWM experiments, and the latency to reach the escape platform was recorded and analysed. For the acquisition trials, a repeated measures AVOVA (treatment group by trials) was used. For the probe trials, a repeated measures AVOVA (treatment group by probe session) was used, with order was a covariant. If the effect of order was found to be insignificant, the probe trial data was grouped. A significant main effect or interaction was followed by post-hoc analysis with Bonferroni’s correction for each assumption where appropriate. When statistical analysis showed that sphericity could not be assumed a Greenhouse-Geisser correction was used.
3.2.2 Object recognition task equipment

A black open-topped box was used as the open-field arena (width 50cm, internal height 25cm). The floor of the arena was divided into 25 equal sized squares, making it easy to divide the arena up into zones. Four different geometric toy shapes were used as the objects; a cube, triangle, oblong and a flower. The objects were well secured onto the base using an internal weight and blu-tack. This made the objects sturdy enough for the mice to interact, lean, and climb on them without the objects moving.

Using the Noldus Ethovision software, movement and object interaction were recorded for each trial and the data processed using SPSS. Animals would be considered as interacting with an object once they were within a 2 cm radius of the object. Data was expressed as both the total duration spent interacting with an object, or as a percentage of the total duration spent with all the objects.

Trials were performed after AIE treatment (see 2.1.3.1). Acquisition of the object recognition task was assessed using a repeated measures ANOVA that was performed on trials 2 – 7, this tested for learning in the task and any bias in object preference. Where post-hoc analysis was used Bonferroni’s correction was used for each assumption. Probe trial data was expressed as the difference between two trials’ percentage object interaction duration, i.e. the spatial relocation was assessed by the change in interaction towards the relocated object in the trials immediately before, and after the relocation.

3.2.3 Locomotion cylindrical runways

Sixteen cylindrical runways (25cm height, 25cm width, and 80cm circumference of the outer wall, with an internal cylinder with width 11cm) were placed on a translucent Perspex table. Above the cylinders were two strip lights and four lamps for lighting, other than mentioned the room was completely isolated from all external light and noise. All animals followed the treatment protocol used in the MWM experiments (see Fig.3.2). The animals were videoed from below to avoid parallax errors and the data was analysed using custom written MatLab programs written by John Anderson & Pete Clifton (2005) which provided estimates of angular velocity and forward locomotion that mimicked those described by Mead & Stephens (Mead and Stephens, 1999).
3.3 Results

3.3.1 The effects of AIE on MWM learning strategy

3.3.1.1 Specific Methods

This experiment had the following group AIE (N = 9) and Control (N = 9). AIE treatment was administered as described in 2.1.3.1, a week of no treatment was allowed for both groups to remove the effects of withdrawal in the AIE group. Training: Animals were trained on the MWM in daily single trial sessions. The escape platform location was held constant for each animal across trials but was counterbalanced for location between animals. The pool entry position was held at the south position all animals. Within each trial, animals were allowed a maximum of 60s to locate and climb upon the escape platform. Probe trials: To explore the strategy that the animals were using to solve the MWM, 2 probe trials were used. These probe trials were counterbalanced for first test across treatment groups. Each probe trial was performed after either a 2g/kg ethanol or saline injection 30 minutes prior to testing. Probe trial A: Vector/head direction strategy: A black curtain was suspended around the pool to remove the use of extra-maze cues which are usually used to aid navigation to the escape platform. All mice were placed in the south entry point and platform location remained the same as it was for their training. To complete this trial the mice have to navigate using internal cues only. Probe trial B: Integration of extra-maze spatial cues: In this probe trial, all extra-maze cues were available but the mice were placed into the pool at the opposite direction than they were trained on (north), the platform position remained the same as it was during training. In this trial the mice have to recognise where they are in the environment and adjust to locate the platform.

3.3.1.2 Results

Before analysis took place the effect of probe trial order was assessed. There were no significant differences found between the Control and AIE treatment groups (treatment by probe trial interaction, p > 0.05), and no difference in latency scores between the two probe trials (main effect of probe trial, p > 0.05) (Fig 3.1). Since the effect of order was found to be insignificant, data from both days were grouped as a single probe trial.
Both groups were able to learn the MWM (main effect of trial, p < 0.05), but showed no significant differences between the groups (treatment by trial, p > 0.05). Post-hoc analysis revealed that the time taken to reach the escape platform was significantly quicker from trial 4 onwards when compared with trial 1 (p < 0.05) (Fig 3.1).

3.3.1.3 Interim Conclusions

These results indicate that AIE treatment did not affect the acquisition of the MWM. Additionally, probe trials where either direction or integration of extra-maze cues navigation strategies were blocked, yielded no difference in performance. Interestingly, the saline treated controls were unaffected by the ethanol injected probe trials. However, greater variability on probe trials may indicate individual difference.

This has demonstrated that AIE treatment followed by drug-free week resulted in no difference in MWM performance when compared to saline treatment mice. However, it is important to model for how binge drinkers actually drink and often this includes learning during binge periods. Therefore the next experiment investigated the effects that AIE treatment has on spatial learning and memory whilst intoxicated/drug free during the training/testing period.
Figure 3.1. Acquisition and probe trials of the effects of AIE on MWM learning strategy experiment. (Left) Latency (s) to reach the escape platform during training and probe trials. Both groups demonstrated that they had learnt the water maze by reducing their latencies ($F_7, 126 = 9.62, p < 0.05$), but there was no between group difference ($F_7, 126 = 0.927, p > 0.05$). Time taken to reach the escape platform was significantly quicker from trial 4 onwards ($T_{19} = 4.2, p < 0.05$). (Right) Spatial and direction strategy probe trials. Score was derived by subtracting the latency of the final training day for both probe trials. No differences were found in latency between probe trials ($F_1, 18 = 1.162, p > 0.05$), and no difference between treatment groups (treatment by probe trial: $F_{1,18} = 0.026, p > 0.05$). Error bars are ±SEM.
3.3.2 Investigation of AIE treatment during the acquisition of the MWM

3.3.2.1 Specific Methods

This experiment adapted the AIE treatment slightly, mice were randomly allocated to one of three groups: the Control group (N = 11) who received equivalent saline AIE starting PND 48. These animals were tested in the MWM on treatment days, 30 minutes after saline injection. There were 2 groups that used the altered AIE treatment. The AIE EtOH-acq group followed the AIE protocol starting PND 48. These animals were treated with ethanol 30 minutes prior to being placed in the MWM. The AIE SAL-acq group followed the AIE protocol starting PND 46. These animals were treated with saline 30 minutes prior to being placed in the MWM. And received their ethanol injections on non-training days (Fig 3.2).

Habituation: All animals were habituated to the MWM for a single trial on PND 45, and followed protocol outlined in 3.2.1. Acquisition: MWM training began on PND 48. 30 minutes prior to the start of each training session mice were injected with either saline (control group and AIE sal-acq) or ethanol (AIE EtOH-acq). Each session consisted of four training trials, one from each entry point (N, S, E and W). The escape platform location was held constant for each animal across all trials but was counterbalanced between animals.

Probe trials: Probe trials started 2 days after the final training day and again one day after that (see Fig 3.2). Two probe trials were used, a saline and ethanol (2g/kg) injected probe trial. Both trials were performed by each group, with the order counter-balanced between groups. During the probe trials the escape platform was removed, the animal was placed into the pool facing the wall and their movement was then recorded for 60s. The subjects were assessed as to how accurate and how often they in the location where the platform would have been in training

Latency to reach the escape platform was averaged across each session (4 trials) and analysed using a repeated measure ANOVA (treatment group by session) (SPSS). Three key variables were measured during the probe trials; the duration of the subject in each zone (NE, SE, SW, and NW), the frequency of “platform position” crossing, and the total distance travelled. Platform crossings were recorded as when the subject passed through the pseudo platform position (the platform was removed for the probe trials) in
the position of all the quadrants. The zones of platform positions were categorised into
one of three categories, correct, adjacent and opposite. The correct zone was where the
platform was located during training. The opposite is the zone directly opposite the
correct zone. The adjacent category is the combination of both adjacent zones.
Figure 3.2. Injection, training and testing protocol for the investigation of AIE treatment during the acquisition of the MWM experiment. The control group received saline injections 30 minutes before training on each training day. The AIE EtOH-acq group received ethanol 30 minutes before training on each training day. The AIE SAL-acq group received ethanol on non-training days. They also received saline injections 30 minutes before training on each training day. Each probe trial test day split the treatment so that half of each group received ethanol or saline.
3.3.2.2 Results

The effect of AIE whilst training on acquisition of the MWM

Analysis of the first day show that even after a single treatment the AIE EtOH-acq group are already performing more poorly (p < 0.05), and continue to do so as the trials progress (Fig 3.3). A repeated measures ANOVA revealed a significant decrease in latency to reach the platform across sessions over all groups (main effect of session: p < 0.05). The rate of acquisition of the task also varied between the treatment groups as shown by a significant interaction between treatment group and session (p < 0.05) (Fig 3.3). Post-hoc analysis of this interaction revealed that all three treatment groups showed an individual significant decrease in latency to reach the escape platform across sessions, showing that the animals were able to acquire the task irrespective of treatment group (Control: p < 0.05; AIE sal-acq: p < 0.05; AIE EtOH-acq: p < 0.05). When performance on each session was compared, the AIE EtOH-acq group were significantly slower at reaching the platform compared with both the Control and AIE sal-acq groups (p < 0.05) followed by t-tests (Control v AIE sal-acq, p > 0.05; control v AIE EtOH-acq, p < 0.05; AIE sal-acq v AIE EtOH-acq, p < 0.05).

Percentage time spent in each quadrant

To test for spatial memory in the probe trials the escape platform was removed and the time spent in each quadrant during 60s was analysed (Fig 3.4). In this experiment a planned comparison was made between groups and correct platform location, treating quadrant or platform crossing as either correct or incorrect.

A repeated measures ANOVA revealed a significant interaction between treatment group and quadrant in the saline probe trial (p < 0.05). When the percentage of time spent in the correct quadrant was then analysed in the saline probe trial, there was a significant main effect of treatment group (p < 0.05), with the AIE EtOH-acq group spending significantly less time in the correct quadrant on probe trials when compared to the Control or AIE sal-acq groups. There was no difference in quadrant preference between the AIE sal-acq and Control groups, with both spending the longest duration in the correct quadrant (Control v AIE EtOH-acq, p < 0.05; AIE sal-acq v AIE EtOH-acq, p < 0.05; Control v AIE sal-acq, p > 0.05). There was no treatment group by quadrant interaction in the ethanol probe trial (p > 0.05). However, a planned comparison was made to
compare treatment group by correct quadrant between probe trials. A repeated measures ANOVA revealed that there was no difference in the duration spent in the correct zone and probe trial (p > 0.05), suggesting that the acute ethanol injection did not significantly impair performance in any group.

**Percentage time spent in each quadrant**

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A repeated measures ANOVA revealed a significant interaction between treatment group and quadrant in the saline probe trial (p < 0.05). When the percentage of time spent in the correct quadrant was then analysed in the saline probe trial, there was a significant main effect of treatment group (p < 0.05), with the AIE EtOH-acq group spending significantly less time in the correct quadrant on probe trials when compared to the Control or AIE sal-acq groups. There was no difference in quadrant preference between the AIE sal-acq and Control groups, with both spending the longest duration in the correct quadrant (Control v AIE EtOH-acq, p < 0.05; AIE sal-acq v AIE EtOH-acq, p < 0.05; Control v AIE sal-acq, p > 0.05). There was no treatment group by quadrant interaction in the ethanol probe trial (p > 0.05). However, a planned comparison was made to compare treatment group by correct quadrant between probe trials. A repeated measures ANOVA revealed that there was no difference in the duration spent in the correct zone and probe trial (p > 0.05), suggesting that the acute ethanol injection did not significantly impair performance in any group.
Figure 3.3. The effects of AIE treatment performed during the acquisition of the MWM. All groups showed an increase in performance indicated by a decrease in latency to reach the escape platform (main effect of session: $F_{7, 203} = 28.15, p < 0.05$), however even on the first performance was not matched between groups as indicated by the * ($F_{2, 33} = 8.187, p < 0.05$). There was a significant interaction between the group’s performance over the session (group by session interaction: $F_{14, 203} = 2.83, p < 0.05$). Each individual group was shown to learn the maze (Saline: $F_{7, 70} = 24.92, p < 0.05$; AIE sal-acq: $F_{7, 70} = 28.85, p < 0.05$; AIE EtoH-acq: $F_{7, 63} = 4.757, p < 0.05$). The AIE EtoH-acq group showed much poorer performance than either other group ($F_{2, 31} = 8.0, p < 0.05$ followed by t-tests. Control v AIE sal-acq, $t_{20} = 1.9, p > 0.05$; Control v AIE EtoH-acq, $t_{19} = 3.46, p < 0.05$; AIE EtoH-acq v AIE sal-acq, $t_{19} = 2.45, p < 0.05$). Error bars indicate ±SEM.
Figure 3.4. Percentage of total duration spent in each quadrant for both the saline (Left) and ethanol (Right) probe trials of the MWM. The correct quadrant contained the location of the platform during the training trials. The adjacent quadrant is sum of both quadrants 90°c either side of the correct quadrant. The opposite quadrant is the furthest quadrant from the target platform. There was a significant effect between treatments in the saline probe trial ($F_{4, 60} = 7.203, p < 0.05$). The percentage time spent in the correct quadrant revealed a between group difference in the saline probe trial ($F_{2, 32} = 12.777$). With the AIE EtoH-acq group spending significantly less time in the correct quadrant as indicated by * (Saline v AIE EtoH-acq, $T_{20} = 4.742, p < 0.05$; AIE sal-acq v AIE EtoH-acq, $T_{19} = 4.417$; Saline v AIE sal-acq, $T_{21} = 0.284, p > 0.5$). The ethanol probe trial showed no significant difference in correct quadrant between groups ($F_{4, 60} = 0.922, p > 0.05$). There was no difference the duration spent in the correct and probe trial ($F_{2, 30} = 0.652, p > 0.05$). Error bars indicate ±SEM.
Platform crossing during the probe trials

A complementary measure to that of quadrant duration is to analyse the frequency that the mouse crosses the location where the platform would have been, essentially reducing quadrant down to the size of the platform (Fig 3.5).

A repeated measures ANOVA of the saline probe trial revealed a group by platform location interaction (p < 0.05) as well as a main effect of platform location (p < 0.05). When the correct platform was analysed alone in the saline probe trial, a significant effect of treatment group was revealed (p < 0.05), post-hoc independent t-tests show that the AIE EtOH-acq group made fewer correct platform crossings during the saline probe trial than Controls (p < 0.05), but not the AIE sal-acq group (p > 0.05). A repeated measures ANOVA of the ethanol probe trial revealed a main effect of platform position (p < 0.05), but not a platform by treatment group interaction. A planned comparison had been made to compare treatment group by correct quadrant between probe trials, this revealed no difference between saline and ethanol probe trials (p > 0.05).

Distance travelled during probe trials

There was no significant difference in distance travelled between treatment group and probe trial (p > 0.05) (Fig 3.6). There was no effect of treatment on distance travelled in each individual probe trial (Saline probe trial, p > 0.5; Ethanol probe trial, p > 0.05). However, the Control group in the ethanol probe trial showed slightly shorter distance travelled, suggesting a sedative effect. However, this was found to be statistically insignificant (Control v AIE sal-acq, p > 0.05; Control v AIE EtOH-acq, p > 0.05).
Figure 3.5. Percentage of total platform crossings for each platform position location (although no platforms were present) for both the saline (Left) and ethanol (Right) probe trials. Analysis of the saline test indicate a treatment by platform location interaction ($F_{3.072, 43.009} = 6.016$, $p < 0.05$), and main effect ($F_{1.536, 43.009} = 22.845$, $p < 0.05$). Analysis of the saline probe trial for correct platform crossings revealed ($F_{2, 30} = 6.042$, $p < 0.05$). Post-hoc analysis showed that the AIE EtOH-acq group showed significantly fewer platform crossings than the Control group as indicated by the * ($T_{18} = 4.224$, $p < 0.05$), but not the AIE sal-acq group ($T_{18} = 1.465$, $p > 0.05$). There was no significant treatment by platform crossing interaction in the ethanol probe trial $F_{2, 28} = 1.146$, $p > 0.05$. Error bars indicate ±SEM.
Figure 3.6. Total distance travelled of each group during both the saline (Top-Left) and ethanol (Top-Right) probe trials. There was no difference between treatment group and probe trial ($F_2, 28 = 1.673, p > 0.05$). All groups travelled the same distance in the saline injected probe trial ($F_2, 30 = 0.034, p > 0.05$). The two ethanol exposed groups show similar levels of movement in the ethanol probe trial, the saline group appear to show a subtle reduction in distance travelled although this is not significant ($F_2, 30 = 2.529, p > 0.05$; Control v AIE sal-acq, $t = 1.793$, df = 20, $p > 0.05$; Control v AIE Eth-acq, $t = 2.046$, df = 18, $p > 0.05$). Examples of an (A) unsuccessful and (B) successful probe trial performance. Circles indicate platform position during training. (A) There is no focus on a particular quadrant and movement appears random. In (B) there are clear signs of focus on one area with little peripheral error. Error bars are $\pm$SEM.
3.3.2.3 *Interim Conclusions*

To conclude, the AIE EtOH-acq group showed an impaired acquisition of the MWM, where the AIE sal-acq group did not. Suggesting that only when ethanol is intoxicating the mouse is it impaired in MWM performance. The AIE EtOH-acq group spent less time in the correct quadrant in the saline probe trial when compared to the AIE sal-acq and Control groups, and performed less correct platform location crossings than Controls, suggesting that prior ethanol exposure whilst training has impacted learning of the MWM. During the ethanol probe trial the groups performed similarly and statistics could not piece the groups apart. It’s possible that in the ethanol probe trial either the ethanol injection reduced the performance of the Control and AIE sal-acq groups, also, being injected with ethanol prior to testing may have slightly improved the performance of the AIE EtOH-acq group.
3.3.3 Investigation of AIE on a spatial and novel object recognition task

3.3.3.1 Specific Methods

All animals were randomly allocated into one of two groups; an AIE group (N =10) starting PND 45, and an equivalent Control group (N =10). Training and testing: Each session consisted of 10 four minute trials. Before each session, each subject received an appropriate injection (i.p) 30 minutes prior to training. Trial 1 was habituation (no objects present). Trials 2 – 7 were training trials and included 3 objects in the same locations throughout each trial. In trials 8 – 9 object 3 was displaced to create a spatial memory test. Trial 10 introduced a novel object instead of object 1 and maintained the previous change of object 3. Each trial started by placing the animal into the centre of the arena. Movement and object interaction was recorded for 4 minutes before the animal was removed and placed into a holding cage for an ITI of 4 minutes. After which the animal was placed back into the maze for the second trial (Fig 3.7).
Figure 3.7 A diagram of the trials and the changes in the object recognition task. The shapes and colours represent the actual shape and colour in the experiment. (Top-Left) Habituation task lasted 1 trial and had no objects in the arena. All subjects are allowed to roam for the trial to experience and habituate to the arena. (Top-Right) These training trials occurred between and including trials 2 and 7, there were three shapes in the arena, a green cube, a blue oblong and a yellow triangle. The subjects actively explore and become familiar with these objects and their positioning. (Bottom-Left) The relocation trials 8 and 9 test spatial memory. The yellow triangle is relocated to another position and the subject is allowed to explore this. (Bottom-Right) The novel test replaced the green cube with a new object, the red flower shape.
3.3.3.2 Results

Training: There was no significant difference in the total amount of time that the animals spent in contact with the objects during training (trial by object by group interaction, p > 0.05), or in the total distance travelled (trial by distance by group interaction, p > 0.05) (Fig 3.8). This is important because for any exploration/interaction based task there needs to be a close relationship between groups in the amount of time they spent exploring the objects.

Spatial memory test: In trial 8, object 3 was moved to a novel location allowing spatial memory to be tested. A repeated measures ANOVA revealed an object by treatment group interaction (p < 0.05) (Fig 3.9). It was found that the AIE group shifted their attention towards the spatially relocated object, object 3, in trial 8 when compared with trial 7, and spent a greater percentage of their overall interaction with the relocated object than the controls (p < 0.05). A test of perseverance of the attention shift towards the relocated object, was conducted in trial 9. Analysis showed that the shift in attention was not preserved into this trial (main effect of object, p > 0.05; object by group interaction, p > 0.05; t-test, p > 0.05). Suggesting that either the mice were now familiar with the object or that the significant result in trial 8 was a false positive (i.e. a Type 1 statistical error).

Novel object test: In trial 10 object 1 was replaced with a novel object allowing non-spatial memory to be tested. There was no significant effect seen by replacing an existing object with a novel object when compared to the previous trial (p > 0.05).

3.3.3.3 Interim Conclusions

There was no object bias or difference in attention towards the objects between the groups during the habituation trials. The AIE group showed a significant increase in investigation of object 3 in trial 8, where controls showed no such difference. However, this did not persist into trial 9, and the effect in trial 8 may have been a false positive. There was no indication that either group shifted attention towards the novel object test, object 1 in trial 10. The data is limited however due to the saline injected controls showing no difference in behaviour in either the spatial or non-spatial tasks.
Figure. 3.8. Results from the training trials in the object recognition task. During the training trials the subjects are placed into the arena that contains 3 different objects (1-3). The training trials are designed to allow the subject time to familiarise themselves with the objects before the objects are relocated or replaced. (Top-Left) Duration spent interacting with object 1. (Top-Right) Duration spent interacting with object 2. (Bottom-Left) Duration spent interacting with object 3. (Bottom-Right) The total distance travelled in each trial. There was no significant interaction between treatment, object and trial during the training trials ($F_{10, 150} = 0.836, p > 0.05$), or in the total distance travelled in each trial between group ($F_{5, 75} = 0.854, p > 0.05$). Error bars indicate ±SEM.
Figure 3.9. The investigation of AIE on a spatial and novel object recognition task. (Top-Left) The difference in object interaction between trials 7 and 8. For trial 8 object 3 was spatially relocated. Overall there was a group by object interaction ($F_{2, 30} = 3.736, p < 0.05$), and the AIE group increase their attention towards the spatially relocated group as indicated by the * ($F_{1, 16} = 5.844, p < 0.05$; $T_{15} = 2.417, p < 0.05$). (Top-Right) Difference in object interaction between trials 7 and 9. The spatial relocation of object 3 continued in this trial, but the AIE group’s attention did not (main effect of object, $F_{2, 30} = 2.792, p > 0.05$; object by group interaction, $F_{2, 30} = 2.344, p > 0.05$; $T_{15} = 1.676, p > 0.05$). (Bottom-Right) Difference in object interaction between trials 9 and 10. In trial 10, object 1 is replaced by a novel object. Neither group increased attention towards the novel object ($F_{2, 30} = 1.076, p > 0.05$). Error bars are ±SEM.
3.3.4 Locomotor activity

Administration of ethanol can lead to unwanted side-effects, including motor impairment. Therefore, it was important to consider the effects that ethanol had on motor function and whether this could have led to impaired performance in the AIE EtOH-acq group from the Investigation of AIE treatment during the acquisition of the MWM experiment.

3.3.4.1 Specific Methods

The locomotor activity experiment consisted of 2 groups, an AIE group (N = 8) and a Control group (N = 8) that followed the same protocol as in the experiment Investigation of AIE treatment during the acquisition of the MWM but injected with saline. **Habituation**: A single habituation day was performed on PND 45. All mice were placed in the locomotor cylinders for 1 hour, under normal experimental conditions, to allow the animal to become familiar with the surroundings. **Sham day**: A single sham-injection day was performed on PND 46 to habituate the animals with being injected and placed into the arena. 30 minutes before being placed into the arena, all animals received saline injections. **Testing**: Before each test-trial all mice were injected with either ethanol (2g/kg) (i.p.) or equivolume saline, 30 minutes before being placed in the locomotor cylinders. As soon as they were placed into the chambers their movement was recorded for 60 minutes.

The results from the experiment were analysed in two different time bins, and measured the distance moved by each subject during the trial. The first 5 minutes, and total duration of each trial session was analysed for differences in distance travelled between the treatment groups. The first 5 minutes was analysed as this would fit best with the AIE EtOH-acq group. Repeated measures ANOVA (treatment by trial) were used to analyse the testing days, with post-hoc t-tests. Bonferroni’s correction was used for each assumption. In total there were 10 trials, 1 habituation, 1 sham and 8 test-trials that were performed on a 2-day-on 2-day-off basis as the AIE.

3.3.4.2 Results

Analysis of the sham day revealed that there was no bias in the performance before experimentation (p > 0.05). A repeated measures ANOVA indicated no difference between groups and sessions (p > 0.05). Day 1’s trial is the first time the AIE group are
exposed to ethanol, although the AIE group showed somewhat higher distance travelled within the first 5 minutes the effect was insignificant ($p > 0.05$), and any difference between groups dissipated in the next 3 trials. The remaining trials showed a general increasing trend in both groups, with only the AIE group showing slightly greater distances travelled, although statistically insignificant ($p > 0.05$). However in both group there was a significant increase in movement between test days 6 and 9 ($p < 0.05$).

The total distance travelled during the whole trial was also analysed (Fig 3.10). The overall pattern is similar to the first 5 minutes, and a main effect of trial was observed ($p < 0.05$), but not a trial by treatment interaction ($p > 0.05$) with no bias in the sham ($p > 0.05$).

### 3.3.4.3 Interim Conclusions

Overall the AIE-treated and saline groups did not display much difference in locomotion. What was clearly noticeable was the greater distance travelled in the sham day, which could be likely explained by a novelty effect. Although statistically insignificant, there was a tendency for the AIE-treated group to have a greater locomotor activity during the first 5 minutes of the experiment, when compared to the saline group. However, over the whole 1-hour testing period the AIE-treated group were generally displaying lower locomotor levels.
Figure 3.10. The effects of AIE on locomotor activity. (Left) The distance travelled in the first 5 minutes of each locomotor trial. The x axis shows the day which the trial occurred, which reflects the AIE procedure. The sham day shows the highest level of movement in both groups in the first 5 minutes of any trial though insignificant between groups ($T_{14} = 0.621$, p > 0.05). Starting from day 1 although the AIE group appear to show a greater movement than the Saline group this was statistically insignificant ($T_{14} = 2.849$, p > 0.05). Between test days 1 and 6 the both groups show a general decline in movement until day 4. Over the remaining test days the AIE group appears to increase locomotion when compared to the saline group. Statistics showed a main effect of trial day and not a trial by treatment interaction (main effect, $F_{1.874, 26.240} = 7.451$, P < 0.05; treatment by trial, $F_{1.874, 26.240} = 1.388$, p > 0.05). * indicate paired t-test results between the days 6 and 9 in the saline group ($T_{7} = 5.034$, p < 0.05) and the AIE group ($T_{7} = 5.015$, p < 0.05). (Right) The total distance travelled in each 60 minute test day. Again the sham trial showed the greatest distance travelled by both groups of any trial with no difference between groups ($T_{14} = 0.152$, p > 0.05). Between test days 2 and 5 the ethanol group appear to show lower overall distance moved than the saline group. However, due to the repeated measures ANOVA indicating no interaction (treatment by trial, $F_{2.836, 39.697} = 0.815$, P > 0.05) it would not be valid to perform t-tests (even if an interaction was assumed, the t-tests would report negative due to the number of assumptions). However, a main effect of trial was revealed (main effect of trial, $F_{2.836, 39.697} = 2.942$, p < 0.05) and the saline group appear to have travelled greater distances in the later test days. Error bars indicate ±SEM.
3.4 Discussion

3.4.1 The effects of AIE on spatial memory in adulthood

This chapter explored the effects of AIE treatment on spatial memory in two different experiments (“The effects of AIE on MWM learning strategy” and “Investigation of AIE on a spatial and novel object recognition task”). In both of these experiments the AIE treatment did not appear to impair spatial memory. Moreover, in the OR task, performance in the spatial memory task was enhanced. This result is supported by other findings in the literature that also used similar dosing and binge-like procedures, and failed to show a subsequent effect on MWM performance and spatial memory (Boulouard et al., 2002, Schulteis et al., 2008, Acheson et al., 2013, Silvers et al., 2003b, Silvers et al., 2006, Tokunaga et al., 2006, White et al., 2000a). This suggests that AIE treatments in rodents is insufficient to cause significant spatial memory impairments in later life, similar to human binge drinking adolescents (Tokunaga et al., 2006). This could suggest that the deficits seen in humans are from some other cause, or, a more likely answer is that it is very difficult to model human adolescent binge drinking in rodents, whose adolescent period is, at maximum, 30 days.

Donald Hebb demonstrated that if a task could be learned using spatial or non-spatial techniques then rodents would preferentially use a spatial strategy to acquire the task (Hebb, 1949), this may reflect a shift towards the increased use of HD cells (Vorhees and Williams, 2014, O'Keefe, 2007). If the AIE treatment did result in deficits in spatial memory (place cells), these deficits may have not have been expressed because the task may have been learnt through non-spatial memory techniques, such as using subtle visual cues (such as lighting levels), or a greater reliance on HD cells. With this reasoning the AIE group in the effects of AIE on MWM learning strategy experiment should have performed the non-spatial probe trial better than controls. However, there was no difference in performance or preference of any particular between AIE treated and control groups when in the non-spatial probe trial.

In the object recognition task the AIE treatment did not produce any bias in exploration of the objects during the training phase when compared to Controls. Although not statistically significant, the AIE group did appear to show a slight reduction in overall activity, in particular across the last few training trials. This was investigated as it was felt that a reduction in movement could result in less object exploration. However, the total
number of object interactions during training was well matched. This therefore allows the conclusion that the AIE treatment did not impair object exploration during the training period.

To investigate spatial memory in the object recognition task object 3 was relocated to an adjacent, previously empty space. The AIE group increased their interaction with the relocated object and reduced interaction with the other objects which remained in the same location. Whereas the Control group interacted equally with all objects, and demonstrated no preference for any object. This preferential shift towards the spatially relocated object indicated a facilitation of spatial memory in the AIE treated group. This is an interesting and unexpected result that contrasted with the majority of relevant literature (Brooks et al., 2002, Ryabinin et al., 2002, Spanos et al., 2012). Others have showed spatial memory impairments in adult male Wistar rats by injecting as little as a single 2g/kg injection per week with sufficient rest before testing in an OR task (Garcia-Moreno and Cimadevilla, 2012). In another study, male Wistar rats that received an ethanol binge-like treatment showed impaired spatial memory one week after treatment (Cippitelli et al., 2010). However the high concentration and lengthy periods of ethanol treatment used in their study was designed to be sufficient to cause ethanol dependence, whereas the AIE treatment used here was designed to better reflect human binge drinking, which does not typically result in ethanol dependence.

Similar to hippocampal lesions, ethanol has been shown to not only inhibit spatial memory but also to facilitate non-spatial memory (Novier et al., 2012). Suggesting that ethanol can be altering how or even where information is processed. For example, a task that is typically hippocampal dependent could be solved by an entorhinal/perirhinal process. In the example of the object recognition task, this could have improved performance (Parron and Save, 2004, Bartko et al., 2007, Norman and Eacott, 2004, Mumby and Pinel, 1994, Ennaceur et al., 1996, Bussey et al., 1999). However, this does seem an unusual result that did not persist into trial 9, and as such may well be a type 1 error.

Trial 10 involved a novel object, non-spatial memory test, where object 1 was replaced with a novel object. Both the AIE and Control groups did not increase their attention towards this novel change, suggesting that non-spatial memory was not impaired by AIE treatment. Although it cannot be ruled out that it did impair novel object recognition as the Controls did not perform the task as expected. However, it has been noted from other studies that acute ethanol (2.4g/kg) administration in male C57BL/6J mice, decreased
exploration of the novel object (Ryabinin et al., 2002). And even doses as low as 1g/kg ethanol can inhibit the exploration of the novel object in adolescents, but not in adult C57BL/6J mice (Spanos et al., 2012). However, these both concern acute ethanol and AIE treatments have been shown to show less behavioural abnormalities after a week drug-free, than during an acute ethanol administration (Sanchez-Roige et al., 2014, Rodriguez-Arias et al., 2011, Pascual et al., 2007, Pascual et al., 2009, Cippitelli et al., 2010). However, since ethanol affects novel object recognition in adolescents at lower concentrations than adults, it could be possible that AIE treatment could lead to deficits in novel object recognition in further studies.

It should be taken into consideration when comparing the AIE procedure here to others in the literature that the protocol used throughout this thesis attempted to better reflect typical human heavy, but not addicted or pathological drinking. In this case, it could be argued that the presented AIE treatment is less severe than other treatments that did produce deficits in spatial memory. This may reflect an interesting treatment/dose-dependent effect on spatial memory.

3.4.2 Tests of spatial memory during the course of AIE treatment

The AIE EtOH-acq group were trained on the MWM during treatment in the Investigation of AIE treatment during the acquisition of the MWM experiment. This group clearly showed impaired acquisition of the MWM when compared to the AIE sal-acq and Control groups. Other studies have observed similar results, showing a dose-dependent effect of ethanol on spatial learning in male adolescent Long Evans rats in the MWM. Where injections of 2.5g/kg ethanol 30 minutes before training in the MWM impaired acquisition of the task, and 0.5g/kg did not (Acheson et al., 2001).

The AIE EtOH-acq group also displayed deficits (when compared to the other groups) in the saline probe trial. These effects are not surprising, as the acquisition of the task was generally poor in this group. However, in the ethanol probe trial these deficits were not as striking, with the AIE EtOH-acq group performing at roughly the same level as the AIE sal-acq and Control group. This could be interpreted in a number of ways. One possibility is that due to the ethanol injection the AIE sal-acq and Control groups performed, (insignificantly) slightly worse. This would narrow the gap between the AIE EtOH-acq group and the others. And although not particularly evident in analysis of the correct quadrant (Fig 3.4), there is a subtle reduction in the Control group in platform crosses
(Fig 3.5). This may reflect that acute ethanol, although not enough to impair the well-learnt spatial memory, is perhaps strong enough to affect the accuracy as the platform measure was much smaller than the zone. Another possibility is that the AIE EtOH-acq group may show signs of a state-dependent effect (Shulz et al., 2000, Goodwin et al., 1969) from learning, as the conditions in testing are the same as they were in training, and this may have aided their performance in the ethanol probe trial. Although it should be noted that the AIE EtOH-acq group did not perform the ethanol probe trial statistically better than it performed the saline probe trial, if the AIE EtOH-acq group had performed better in the ethanol probe trial it may suggest the development of a tolerance. It is likely that all of these factors could have played their role in the ethanol probe trial, but due to the strong memory the mice had of the platform location it was difficult for it to become impaired.

The AIE sal-acq group were trained on non-drug days in the Investigation of AIE treatment during the acquisition of the MWM experiment. This group showed no impairment of acquisition of the MWM when compared to Controls, or impairment in either saline or ethanol probe trials. This finding is supported by a study of male adolescent Sprague Dawley rats that were trained in the MWM. After training was complete they received CIE vapour treatment for 4 days, on a subsequent test CIE was shown to not inhibit spatial memory (Van Skike et al., 2012). Another study showed that CIE treatment conducted on non-training days in Sprague Dawley rats, shared the result reported in this chapter, and did not show any impairment of acquisition. However, they also showed a tolerance to the ethanol probe trial (Silvers et al., 2006). It was inconclusive as to whether the AIE sal-acq group experienced tolerance in the ethanol probe trial of the MWM because the Control group did not show any significant signs of impairment, and the Control group could not have developed tolerance to ethanol. However, it has already been mentioned that their accuracy may have been subtly, insignificantly impaired. This provides further evidence that AIE treatment even without a period of rest to avoid acute withdrawal, still does not develop spatial memory deficits in the MWM in C57BL/6J mice.

3.4.3 Overtraining of spatial memory and AIE

One possible reason why AIE didn’t appear to impair spatial memory performance in the MWM mazes is that the mice could have been too well trained. This could have reflected over-learning/training in, particularly the AIE Sal-acq and Control group from the
Investigation of AIE treatment during the acquisition of the MWM experiment, but also both groups from The effects of AIE on MWM learning strategy. It has been hypothesised that NMDA receptors are required in the early acquisition of the MWM and possibly other spatial memory tasks (Nakazawa et al., 2003, Nakazawa et al., 2002, Place et al., 2012). But they may not be needed once the memory has become sufficiently consolidated. If training continues so that a spatial memory is less dependent on new learning it may be NMDA independent (Nakazawa et al., 2004, Moser and Moser, 2000, Place et al., 2012). This suggests that the place and head direction cells are acting as way pointers to the neocortex (or elsewhere) and the spatial processing is less hippocampal-dependent (O’Keefe, 2007). Therefore these groups may be too trained and the ethanol treatments are unable to impair performance as the memory is too consolidated. The size of the MWM is correlated with its difficulty (Morris, 1984), the larger the surface area the more the subject has to search in order to find the escape platform, making it harder to form a spatial map, and requiring more processing from the place cells in the hippocampus (Vorhees and Williams, 2006). Perhaps using a larger pool in these experiment would allow for a tougher assessment of spatial memory, and less of what could have been a highly consolidated memory.

3.4.4 Effects of ethanol on locomotion, general movement and motivation

In these experiments it is possible that the AIE EtOH-acq group increased the latency to reach the platform due to an impairment of movement rather than an impairment of spatial memory. To measure the effect of distance travelled after ethanol injections a locomotor experiment was performed. In this experiment the injection protocol mirrored the AIE treatment. The result from day 1 of the locomotion experiment showed higher movement levels when compared to the following days. This could be due to the novelty of the new surroundings, as it is known that mice like to explore novel environments (Vorhees and Williams, 2014, Sanderson and Bannerman, 2010). In the days following it appears that, if anything, ethanol is acting as a stimulant during the early stages of the locomotor trial (first 5 minutes), and then as a sedative later in the trial. This could mirror the typical rising and falling phases of ethanol use (Deitrich et al., 1989), where shortly after ethanol use or injection, ethanol acts as a stimulant but then becomes more sedative with time (Julien, 2011). Moreover, the distance travelled in MWM probe trials show no real differences between the groups. However, the locomotor experiment measured how far the mice moved after an injection of ethanol or saline. Even if the total
distance travelled are comparable between the groups this does not necessarily mean that their efficiency or agility was not impaired. There is at least one example suggesting that ethanol injection produced an inefficiency of movement in rodents in the MWM (Acheson et al., 2013). Moreover, ethanol-induced impairments in motor coordination have been proposed for MWM results that apparently indicate deficits in spatial memory (Schulteis et al., 2008).

A foot pattern study may have better reflected accuracy during swim performance. One such study confirmed that the same AIE treatment that was used in this study produced minor differences in foot placement and the increased the failure rate when tasked with walking across a beam in adulthood (Forbes et al., 2013). It is suggested that this is due to a neurodegenerative effect on the purkinje cells of the cerebellum (Forbes et al., 2013). It is interesting that this result revealed a deficit between AIE and saline treatments. However, the study did not investigate the effects of ethanol whilst intoxicated, which from the experiments in this chapter clearly had a much greater effect.

Somewhat related is the issue of motivation. Ethanol treatments may be effecting performance by altering the strength of the negative reinforcement (water) to locate the escape platform. One such study hypothesised that ethanol treated rats did not show any particular deficits in cognitive mapping, but when performance deficits were apparent they offered they felt that this reflected the motivational effects of ethanol (Devenport et al., 1989). The MWM is a subtly aversive task (Morris, 1984, Morris et al., 1982, Vorhees and Williams, 2006), where the water acts as a stressor, increasing anxiety. The rodent involved aims to locate the platform and remove the stressor (Vorhees and Williams, 2014). Anxiety itself can modify performance in behavioural tasks and can even alter learning and memory (Silva and Frussa-Filho, 2000). Interestingly though, as ethanol is an anxiolytic shortly after dosage (Kameda et al., 2007, Popovic et al., 2004, Wilson et al., 2004) (arguably anxiety-inducing during withdrawal or hangover) it is possible having a reduction in anxiety as a result of being injected with ethanol could have reduced the amount the subject was stressed by the water and may have resulted in altered behavioural performances (Sircar et al., 2009). For instance, it was noticeable whilst observing the experiments that AIE EtOH-acq group took far more stops when in the pool after ethanol injections than those in the pool under saline conditions. This could be in part due to a decrease in anxiety, possibly to the point where motivation to escape the pool was reduced sufficiently enough to effect escape latencies. A study performing a longitudinal assessment of ethanol treatment in rats showed increased anxiety and a
greater thigmotaxic response, even 120 days after treatment (Santucci et al., 2008). Binge-like and chronic ethanol treatments have been shown to increase anxiety in rats in the long-term (Pandey et al., 2006, Briones and Woods, 2013). Others have argued that reduced anxiety via ethanol may lead to spending more time in the centre of the pool and not circling around the walls (a common behaviour from nervous animals in many behavioural experiments) (Sircar et al., 2009). The same study suggested that this could result in faster MWM latencies, but were unable to confirm this hypothesis (Sircar et al., 2009). The escape platform in the MWM experiments in this chapter were not close to the pool wall, so a thigmotaxic response would have not aided performance. More recent work has suggested that the hippocampus is particularly sensitive to stress and that it can create spatial memory deficits and general dysfunction (Tomar et al., 2015).

Additionally the AIE EtOH-acq group exhibited different swimming behaviours than the AIE sal-acq and Control groups. Behaviours such as floating still rather than moving and more stop and start movements. It could be posited that these mice were less anxious of the water, and therefore less motivated to escape the MWM maze. Occasionally, these mice would bump into the escape platform but would not attempt to climb to platform to escape, and rather continue explore the pool. It is not known whether this is due to decreased anxiety and that the mice want to explore, or that they were not aware that they had come into contact with the escape platform.

The object recognition task was performed after a treatment period and not prior to testing so they would not have been an anxiolytic effect. The ethanol treatment could have resulted in increased anxiety in the mice, and they would show increased thigmotaxic behaviour (Santucci et al., 2008). However this was not the case as both groups interacted with the objects at the same levels. A more recent example showed that AIE resulted in anxiety-like behaviour and a reduction in novel object recognition memory (Vetreno et al., 2015). However, they measured anxiety in the form of thigmotaxis and latency to enter the centre of the open-field maze, which could easily occur at random, or dependent on the pre-exposure/habituation to the open field.
3.4.5 The effects of acute ethanol on spatial memory

Several experiments reported in this chapter used a combination of AIE and acute ethanol treatments to investigate how ethanol affects memory. Acute ethanol in the probe trial appeared to have only minor effects on Saline and AIE sal-acq groups, for instance see Fig 3.1, where the saline treated controls did not become impaired in the probe trials after receiving ethanol. This result contradicts other studies in the literature. One series of studies that used a similar binge model to the AIE treated used here, was performed by Silvers et al (Silvers et al., 2006, Silvers et al., 2003b). In these studies they injected (i.p.) 5g/kg ethanol during early adolescence in SD rats on a one-day-on one-day-off pattern for a total of 10 injections. Training was performed during the binge-treatment period, but on non-injection days (equivalent to the AIE sal-acq group). Their AIE group shared the acquisition finding presented here and that will be discussed in the next section. But they also found an ethanol tolerance of the memory impairing effect of acute ethanol in the probe test. This difference in results is likely to have come from the difference in dosing, with 5g/kg being much greater than 2g/kg, suggesting a dose-dependent effect of AIE on performance in the MWM. More similar acute ethanol doses on adolescents have resulted in impaired spatial memory, but spared non-spatial memory (Berry and Matthews, 2004, Van Skike et al., 2012). However reports are mixed on the effects of ethanol during adolescence. With some reporting that acute ethanol does not differ between adolescents and adults in its impairment of spatial memory (Chin et al., 2011, Novier et al., 2012, Rajendran and Spear, 2004, Hefner and Holmes, 2007).

Other studies have suggested that even with spatial memory significantly impaired mice are still able to learn the MWM, but via a different strategy. They found that CaMKII mutated mice did not depend on using specific external maze cues, and could have learnt the platform's distance from the edge of the pool and located it by swimming around at that distance (Silva et al., 1992a). This could suggest that the task could be completed by using HD cells conveying to the place cells and not dependent on the place cells having a fully built spatial map, implicating a non-spatial performance in the task.

3.4.6 Conclusions

To conclude, in the initial The effects of AIE on MWM learning strategy experiment the ethanol treatment during late adolescence did not produce any difference in acquisition of the water maze in early adulthood. Nor was there any significant difference between
the groups in the spatial and non-spatial probe trials. Therefore in this instance the ethanol treatment did not produce any detectable deficits in spatial memory.

The *Investigation of AIE treatment during the acquisition of the MWM* experiment revealed a similar lack of effect as seen in the first experiment when the ethanol treatment was administered on non-training days. However, if ethanol was administered shortly before each training trial, then an impairment in acquisition became evident. In the probe trials those animals who were treated with ethanol shortly before training trials performed significantly poorer than controls in the saline probe trial. Demonstrating that the poorer learning seen in the AIE-acq group persisted when tested without ethanol. There were no treatment group differences in in the ethanol probe trial. This suggests that in both the controls and AIE Sal-acq groups either the memory was highly consolidated and possibly located in another neural region, or that the ethanol concentration was insufficient to cause a spatial memory deficit.

The *Investigation of AIE on a spatial and novel object recognition task* revealed that the ethanol treatment did not impact on object interaction in the first 7 trials. However during a spatial memory trial the ethanol treated group displayed a preference for the spatially relocated object, suggesting a facilitation of spatial memory. There was no significant effect in the trial in which a novel object was presented.

Finally, the *locomotor activity* experiment did not show significant differences between ethanol and saline prior injection. Though there was a general trend for the ethanol group to travel more during the first 5 minutes, and less overall after 60 minutes. However, locomotor activity measure reflects the distance travelled and not necessarily the lack of motor coordination.

In summary, when ethanol is administered shortly before the water maze and object recognition tasks there is clear impairment of performance. However ethanol treatment using the AIE protocol did not affect performance on the water maze in adulthood, and was associated with a facilitation spatial memory in the object recognition task. It would be interesting to explore how adolescent ethanol treatment and various ethanol application protocols affect hippocampal physiology directly. This can be performed by employing electrophysiological techniques and investigating hippocampal LTP.
Chapter 4 – The effects of adolescent intermittent ethanol, and acute ethanol treatments on hippocampal LTP

Data presented in the previous chapter revealed a clear difference in the performance of spatial memory tasks when ethanol was present at the time of training, when compared to adolescent intermittent ethanol (AIE) treatments in which ethanol was not present at the time of training. This suggests that ethanol could be interacting with the hippocampus in different ways depending on when it is administered, and that acute ethanol treatments may disrupt hippocampal functioning during task training. One direct test of this hypothesis would be to use both types of treatment in conjunction with the hippocampal slice technique to investigate how ethanol affects hippocampal LTP.

The AIE treatments in the previous chapter did not reveal any noticeable deficits in spatial memory. However this is not fully consistent with the wider evidence from rodent models (Sabeti, 2011, Sabeti and Gruol, 2008, Fleming et al., 2013) and humans (Weissenborn and Duka, 2003, Townshend and Duka, 2005, Tapert et al., 2004b). Therefore it remains worthwhile to pursue investigation into the electrophysiological effects that such ethanol treatments have on hippocampal function.

This chapter first builds on the discussion of the hippocampus and LTP presented in the general introduction and provides a review of the literature surrounding the effects acute ethanol and AIE-like and CIE-like treatments have on LTP and hippocampal plasticity. Then 4 experiments are described, the first models the effects of systemic acute ethanol application using hippocampal slices and investigates how ethanol may be affecting hippocampal LTP. The remaining three experiments investigate the effects that AIE treatments have on hippocampal LTP in later life, to investigate whether any long-term physiological effects are evident.
4.1 Introduction

Of all of the regions of the brain where LTP has been studied, the hippocampus has received the greatest amount of attention. The hippocampus is easily accessible for surgical intervention in rodents, and its structure makes it visible to even the untrained eye. These attributes combined with the hippocampus’ critical role in memory functioning, make the hippocampus a great tool for furthering the understanding of how the brain forms and recalls our memories. As discussed in the general introduction, when ethanol is consumed in a binge-like pattern it can result in a variety of impairments, including impairing memory function. One way in which ethanol is thought to induce these memory impairments is by inhibiting LTP, a form of synaptic plasticity associated with memory processing (Tokuda et al., 2013). It has also been posited that when someone drinks excessively and suffers acute memory loss, this could be the action of ethanol inhibiting LTP (Izumi et al., 2005, Allgaier, 2002).

It is known that the hippocampus, and related temporal lobe structures, are critical to human memory from studies such as those of patient H.M (reviewed in (Annese et al., 2014)), and from numerous lesion studies performed in primates and rodents (Morris et al., 1982, Morris, 1984, D’Hooge and De Deyn, 2001, Pearce et al., 1998, Cho et al., 1999). The hippocampus itself has had its physiology extensively studied and characterised and has been used to investigate LTP successfully for many years (Bliss and Gardner-Medwin, 1973, Bliss and Lomo, 1973, Alger and Teyler, 1976). However, LTP and the hippocampus have been mentioned frequently in this thesis so far, the detailed physiology of the hippocampus has not been explored until this point.

4.1.1 The effects of acute ethanol treatment on LTP

Consistent with binge drinking impairing memory and relatively high acute ethanol consumption leading to acute amnesia (‘blackouts’) (Silvers et al., 2003a, White, 2003, Matthews and Silvers, 2004), several studies utilising animal models have found that ethanol can inhibit the formation of LTP in the hippocampus (Sinclair and Lo, 1986, Tokuda et al., 2007, Izumi et al., 2015), for instance, 60mM ethanol inhibits LTP in C57BL/6J mice (Zorumski et al., 2014, Ramachandran et al., 2015).
Ethanol inhibition of LTP is suggested to occur by increasing GABAergic inhibition (Sanna et al., 1993, White et al., 2000b, Izumi et al., 2007), this increase in inhibition, inhibits the depolarisation-dependent NMDA receptor activation (Schummers and Browning, 2001, Ramachandran et al., 2015). Whereas others suggest that ethanol enhances inhibition via a GABA independent mechanism (Fleming et al., 2013). Additionally, ethanol has action on many other neurotransmitter pathways and ion channels, such as the BK potassium channel (Dopico et al., 2014). This, like GABA transmission, could also inhibit the depolarisation needed to NMDA activation. Ethanol could also be acting on NMDA receptors directly (Sabeti, 2011, Sabeti and Gruol, 2008, Mulholland et al., 2009). However, it has also been argued that pharmacologically relevant concentrations of ethanol produce only very modest inhibition of NMDA receptors in the CA1 region, and that these ethanol concentrations cannot antagonises NMDA receptors enough to account for the inhibition of CA1 LTP (Lovinger et al., 1989, Murayama et al., 2006, Schummers et al., 1997, Schummers and Browning, 2001).

There has been growing evidence that other neuromodulators are implicated in the inhibition of LTP by ethanol. Acute ethanol has been shown to increase the levels of the neurosteroid allopregnanolone in CA1 pyramidal neurons, and allopregnanolone has been shown to inhibit hippocampal LTP by itself (Tokuda et al., 2011, Ramachandran et al., 2015), perhaps by potentiating GABAergic transmission (Zorumski et al., 2014, Ramachandran et al., 2015). Ethanol’s secondary metabolites are becoming increasingly implicated in many of the effects of alcohol on the body and a recent study suggests that ethanol metabolites, especially aldehyde, are locally generated by CA1 neurons and may also affect LTP (Tokuda et al., 2013).

The ethanol concentration needed to inhibit LTP in these studies varies between both experimenters and rodent strains, but most report the inhibition of LTP at ethanol concentrations in excess of 50mM in vitro (Morrisett and Swartzwelder, 1993, Roberto et al., 2002, Schummers et al., 1997, Tokuda et al., 2007, Izumi et al., 2007, Randall et al., 1995, Sugiura et al., 1995, Bliss and Collingridge, 1993, Martin et al., 2000, Sinclair and Lo, 1986, Blitzer et al., 1990, Durand and Carlen, 1984). In vivo ethanol concentrations that are known to inhibit memory function in behavioural studies (1g/kg), also blocked LTP induced by θ-burst stimulation (Givens and McMahon, 1995). Additional electrophysiological data from a whole cell current clamp technique on cultured rat hippocampal neurons showed that 44mM ethanol in vitro decreased cell
spiking, the amplitude of the hyperpolarisation and the amplitude of the off-response generated at the termination of a hyperpolarisation pulse (Urrutia and Gruol, 1992).

A key issue with majority of in vitro ethanol LTP studies is the rate at which ethanol is applied to the hippocampal tissue is highly unlikely to occur in vivo in rodents or humans (Perkins et al., 2001, Lange and Voas, 2001). Often in these experiments the ethanol concentration escalates to 50-60mM within a matter of minutes or less (Izumi et al., 2005). To more accurately model the nature of ethanol increases seen in the blood, Tokuda, (2007) using hippocampal slices from adult rats, slowly increased the ethanol concentration from 0mM to 60mM, using multiple concentration steps every 15 minutes. Under these conditions, ethanol failed to inhibit the induction of LTP. Further research demonstrated that this form of plasticity was NMDA receptor-independent, shown by bath applying the NMDA receptor antagonist 2-amino-5 phosphonovalerate (APV) (Tokuda et al., 2011, Tokuda et al., 2007).

4.1.2 How repeated ethanol treatments affect LTP

To study for the effects of binge treatment on hippocampal LTP, ethanol treatments such as adolescent intermittent ethanol (AIE) and chronic intermittent ethanol (CIE) need to be performed in vivo during the life of the animal which is later culled, and examined in vitro. Such an investigation was performed in rats subjected to AIE during early adolescence. In this study rats were exposed to ethanol for 14h per day by vapour inhalation for 12 – 14 days and hippocampal slice recordings were performed 24 hours after the final treatment day. Electrophysiology recordings of hippocampal slices showed a compound of traditional NMDA receptor-dependent LTP, and a new, additional NMDA receptor-independent form of LTP (Sabeti and Gruol, 2008). These two forms of LTP were not mutually exclusive and could be co-expressed to produce a greater output, much like a summation of the two individual forms of LTP. The same series of experiments also highlighted critical age-dependent effect of AIE treatment. For example, if treatment was performed during early adolescence’ (PND 30-45), then the rats showed higher LTP values than controls, when later tested in adulthood. However, if the binge-like treatment was performed during late adolescence’ (PND 45-60) then the rats showed lower levels of LTP when compared with controls, when later tested in adulthood (Sabeti, 2011, Sabeti and Gruol, 2008).
Other groups have also shown age-related differences in the effect of ethanol on hippocampal LTP. LTP was inhibited by low concentration of ethanol (10 or 30mM) in rat hippocampal slices taken during adolescence (PND 30) but not in slices taken during adulthood (PND 90) (Pyapali et al., 1999). Others have also shown that ethanol is a more potent inhibitor of LTP in adolescent hippocampal slices than adult slices (Swartzwelder et al., 1995, Markwiese et al., 1998, Durand and Carlen, 1984). Together these indicate an important age-related effect of intermittent ethanol treatments on the later expression of hippocampal plasticity.

More recent investigation of AIE treatments on hippocampal slices found alterations in tonic current and subsequent sensitivity to ethanol. Moreover these findings were not found in adult IE treated animals, suggesting that the developing adolescent hippocampus is more sensitive to long-term alterations following treatments like AIE (Fleming et al., 2013). There is also growing suggestion that extrasynaptic δ containing GABA receptors, may be implicated in the longer term alterations seen after AIE treatments (Fleming et al., 2013). Others have shown that AIE, CIE and other more chronic ethanol treatments can result in the upregulation of NR2A and NR2B NMDA receptors subunits (Roh et al., 2011, Nagy et al., 2005) (Nelson et al., 2005, Qiang et al., 2007). These upregulations in NMDA subunits are also associated with LTP abnormalities (Roberto et al., 2002). Even acute ethanol exposure can result in the temporary upregulation of NMDA (Roh et al., 2011). However, longer treatments also result in an increase in NMDA receptor mRNA expression (Darstein et al., 2000, Ramachandran et al., 2015). Ethanol treatments are also known to upregulate GluR2 subunits in the hippocampus and (Bruckner et al., 1997). Together this may increase NMDA receptor expression similar to alcohol dependent humans (Julien, 2011).

### 4.1.3 Aims and hypotheses

To summarise, ethanol has a complex action on LTP in the hippocampus, where acutely it can block LTP (Tokuda et al., 2007, Ramachandran et al., 2015, Izumi et al., 2015), but also mediate an ethanol-tolerant form of LTP (Sabeti et al., 2007, Sabeti, 2011, Sabeti and Gruol, 2008). Moreover, AIE and similar protocols (Fleming et al., 2013, Markwiese et al., 1998, Pyapali et al., 1999), suggest that the developing hippocampus may be susceptible to damage as a result of adolescent binge-like drinking.
The aim of the experiments reported in this chapter is to investigate how ethanol affects hippocampal LTP in animals exposed to either acute or AIE. The first experiment investigates how various, more realistic applications of ethanol affect LTP. By using a small chamber it is possible to rapidly increase, or wash-out the ethanol concentration being applied to the hippocampal tissue.

The remaining three experiments investigate the effects of AIE treatment on hippocampal LTP in adulthood. By using the same AIE treatment and protocol it is possible to compare these results with the behavioural effects reported in the previous chapter. Thus the main aims of this chapter are:

1. To investigate the effects that slower increases of ethanol has on hippocampal LTP.
2. To investigate the effects that AIE treatment has on hippocampal LTP in adulthood.
4.2 Methods

4.2.1 Hippocampal slice electrophysiology

4.2.1.1 Field potential recording

A Narishige PC-10 Vertical 2-step puller was set to create 3-5MΩ resistance electrodes from borosilicate capillary tubes purchased from Warner Instruments (outer diameter = 1.5mm, internal diameter = 1.17mm, length = 10cm). A stimulating electrode was fashioned from two pieces of tungsten wire (Advent research materials 99.95% purity, 0.075mm diameter) which was melted to a fine point with the wires separated by capillary tubes.

The hippocampal slices were placed on top of a custom made mesh (ladies tights) that was attached to a small plastic block designed to allow water to flow through it and was placed into the recording chamber. The mesh was slightly slack and would allow for a small drop in height when compared to the plastic block. This allowed for the perfusion fluid to come into contact with the slice. Fluid perfusion was constant, providing the slice with needed ions and oxygen. The perfused fluid was constantly washed off, the fluid was pumped in at one end, and passively exited at the other. This allowed for much better control of ethanol concentrations as the fluid to be perfused could be switched from a 45mM to 60mM solution instantly. It also allowed for ethanol to be removed from the slice almost immediately.

Narishige micromanipulators held the recording electrodes in position. The recording electrode was attached to a Neurolog NL104 preamp via a Digitimer DS2A – MKII stimulator and Neurolog NL100 headstage. This analogue signal was converted into a digital signal using a micro 1401 (CED), which was connected to a computer with the signal 4 software (CED) for analysis.

4.2.1.2 Slice preparation

Hippocampal slices were obtained from mice and cut into 250µm slices with a McIlwain tissue chopper and kept in artificial cerebral spinal fluid (aCSF). A 10x concentration stock of aCSF was prepared 24 hours before experimentation consisting of 1.24M NaCl, 30mM KCl, 260mM NaHCO₃, 12.5mM NaH₂PO₄, 10mM MgSO₄, 100mM D-glucose. On
the day of recording the stock solution was diluted 10 fold and 2mM CaCl₂ was added. The aCSF was warmed using a HAAKE P14 water bath and oxygenated using a carboxygen mixture (95% O₂ / 5% CO₂). The solution was perfused through silicon tubing using a Gilson minipuls 3 peristaltic pump into a custom made recording chamber. All aCSF passed through the chamber with little resistance or wave-impact to the tissue slices and exited the chamber where it was drained into a waste tub. The slices were lit by a Cole Palmer 41723 – Illuminator and viewed using a Motic microscope.

4.2.1.3 Ethanol perfusion protocol

The ethanol protocol in this experiment was adapted from Tokuda et al (Tokuda et al., 2007) who attempted to better model drinking patterns in vivo by slowing increasing the ethanol concentration to 60mM. Clinically, an ethanol concentration of 20mM is considered intoxicated in most countries, with 50 – 60mM ethanol concentrations representing significant intoxication (White, 2003). Most hippocampal slice electrophysiology experiments that investigate the effects of ethanol on LTP raise the concentration to 50 – 60mM almost immediately, even heavy binge drinking does not raise the blood ethanol concentration that rapidly (Lange and Voas, 2001, Perkins et al., 2001). Therefore to create a slower, more realistic increase in ethanol concentration, numerous ethanol concentrations where perfused onto the tissue to mimic a slower, steadier increase in ethanol concentration. For instance, a 2g/kg ethanol injection into a rodent produces ~45mM ethanol concentration 30 minutes after injection (Pascual et al., 2007).

4.2.1.4 Analysis and statistics

Analysis was performed using the Cambridge electronic design (CED) software, Signal. All data is expressed as a percentage of the baseline fEPSP slope%. In experiment 1, the baseline was calculated as the last 5 minutes of the baseline period, which came immediately before the treatment section. In all other experiments baseline was expressed as the mean of the last 30 minutes of the baseline period.

One-way ANOVA’s were used to analyse significance between groups 60 minutes after HFS (LTP60), the end point of the experiment. Where post-hoc analyses used Bonferroni’s correction for multiple comparisons. In some cases a repeated measure ANOVA was used to evaluate the change between PTP (post-tetanic potentiation) and LTP periods.
4.3 Results

4.3.1 Experiment 1: The effects of various acute ethanol protocols on hippocampal LTP

4.3.1.1 Experiment 1: Specific Methods

This experiment investigated the effects of different concentration of ethanol application on hippocampal LTP, where the ethanol concentration was varied prior to induction of LTP. After the stimulus intensity was established, a 60 minutes baseline dataset was recorded, followed by a HFS to induced LTP. After this a further 60 minutes were recorded to measure LTP. All the ethanol treated groups were designed so that the mean concentration of ethanol per minute was as similar as possible during the baseline phase, i.e. one hour of 25mM ethanol equated to half an hour of 50mM ethanol. Five experimental groups were evaluated:

1. The Control group (N = 7, PND 45 – 60). Throughout the recording aCSF was continuously perfused to the recording slice (Fig 4.1 a).

2. The Pyramid group (N = 7, PND 45 – 60) was designed to investigate the effects of a slower application and removal of ethanol, which may better reflect the in-vivo kinetics of ethanol administration. This consisted of a stepped increase and decrease in ethanol concentration during the baseline period. No ethanol was present during the HFS (Fig 4.1 b).

3. The Ramp group (N = 7, PND 45 – 60) was similar to the pyramid group, but used to investigate the effects of having ethanol present at LTP induction. This treatment consisted of a stepped increase in ethanol concentration during the baseline period reaching a maximum of 60mM which was maintained during LTP induction and LTP phase (Fig 4.1 c).

4. The Burst group (N = 6, PND 45 – 60) was designed to investigate the effects of prior exposure of 60mM ethanol on LTP. This immediate onset of 60mM ethanol concentration is used in hippocampal slice studies, but is unlikely to occur in in-
vivo. This group was perfused with 60mM for 30 minutes during the baseline phase (Fig 4.1 d).

5. The Off-On group (N = 7, PND 45 – 60) was designed similar to the burst group, but also measured LTP in the presence of 60mM ethanol. This group were perfused with 60mM ethanol 30 minutes into the baseline period and this concentration was maintained during HFS and until the end of the recording (Fig 4.1 e).

4.3.1.2 Experiment 1: Results

Using multiple perfusion protocols the effects of 60mM ethanol on hippocampal CA1 field potentials was investigated in late adolescent C57BL/6J male mice (Fig 4.2).

The first finding was that the Control group show in increase in EPSP slope% as a result of the induction of LTP by HFS (p < 0.05). In the investigation of the fast onset ethanol protocols, an ANOVA at LTP60, showed that groups Pyramid and Off-On groups experienced different levels of fEPSP slope% (p < 0.05). Subsequent post-doc independent t-tests, showed that the control group displayed greater LTP than the Off-On group (Control v Off-On, p < 0.05), and a trend when compared with the burst group (Control v burst, p = 0.075). Therefore ethanol is inhibiting LTP even when ethanol is absent at the induction of LTP by HFS.

In contrast to the two fast ethanol protocols, by increasing the ethanol concentration more slowly LTP could still be induced, even in slices that had been exposed to 60mM ethanol. The Pyramid group showed similar levels of fEPSP slope% after the induction of LTP by HFS as the Control group. An ANOVA at LTP60 revealed a trend between treatment groups (p = 0.082). Interestingly the Ramp group which shares the Pyramid group’s slow rise in ethanol concentration, but where ethanol is present at the time of HFS, did not show LTP.

4.3.1.3 Experiment 1: Interim Conclusions

These results demonstrate that LTP cannot be induced in the presence of 60mM ethanol, even if ethanol has been removed prior to the HFS. However, if ethanol is slowly raised and then slowly reduced, LTP can be induced.
Figure 4.1. Protocols for the investigation of the effects of various acute ethanol on hippocampal LTP. All recordings form two 1-hour periods, the first is pre-HFS and acts as a baseline period. The second is post-HFS and measures for LTP. a) The control group did not receive any ethanol at any point and was entirely perfused with ACSF alone. b) The Pyramid group slowly increases to 60mM and then slowly decreases to 0mM ethanol during the pre-HFS period. c) The Ramp group slowly increases and then maintains its ethanol concentration at 60mM for the rest of the recording. d) The Burst group applies 60mM ethanol rapidly and washes out ethanol very rapidly during the pre-HFS period. e) The Off-On group applies 60mM ethanol rapidly 30 minutes into the pre-HFS and maintains until the end of the recording.
Figure 4.2 Experiment 1: The effects of various ethanol protocols on hippocampal LTP. Graphs are measuring the % change in fEPSP% between pre- and post-HFS. Post-tetanic potentiation (PTP) and long-term potentiation (LTP) are measured at different times. Top-left: changes in fEPSP slope% after fast-onset ethanol application. The control group was shared between both graphs and showed LTP when compared to baseline (T = 3.241, p < 0.05). Both ethanol applied groups do not show any increase in EPSP slope% and both show lower levels than controls (F = 7.871, p < 0.05) (t-tests (Control vs Off-On, T = 4.044, p < 0.005. Control vs Burst, T = 2.554, p = 0.075)). Bottom-left: changes in EPSP slope% after slow-onset ethanol applications. The control group, displayed a reduction in EPSP slope%. Whereas the Pyramid group, displayed an increase in EPSP slope%, similar to the control group. However, strict statistical analysis could not differentiate between the groups (F = 2.857, p = 0.082). The images in letters A - E are representative images of the pre- and post-tetanic stimulation EPSP slopes. It should be noted that these images are a very small representation of the thousands of spikes used in the analysis and perhaps not accurately reflect the overall groups values.
4.3.2 Experiment 2: The investigation of AIE on hippocampal LTP

4.3.2.1 Experiment 2: Specific Methods

The following 3 experiments investigate the effects of AIE treatment on adult hippocampal LTP. Experiment 2 consisted of 2 groups and AIE group (N = 9) and a saline treated Control group (N = 9). Each animal was either treated during late adolescence with saline or 2g/kg ethanol, for a review of the AIE treatment see section 2.1.3.1. Electrophysiological recordings were made 7 days after the end of each individual mouse’s AIE treatment. All recordings were performed in slices only perfused with aCSF, and consisted of a 60 minute pre-HFS baseline phase and a 60 minute post-HFS LTP phase.

4.3.2.2 Experiment 2: Results

Investigation of the AIE treatment showed that within the first 5 minutes of HFS an increase in fEPSP slope% was seen in both groups. The fEPSP slope% in the AIE group displayed roughly a 70% increase in fEPSP slope% 60 minutes after induction of LTP by HFS when compared to baseline, whereas the Control group displayed roughly a 20% increase in fEPSP slope% 60 minutes after HFS when compared to baseline. Despite an ANOVA at 60 minutes not confirming that the AIE group showed greater LTP levels than controls ($F_{1, 7} = 3.003$, $p > 0.05$), this is an interesting trend (Fig 4.3).

4.3.2.3 Experiment 2: Interim Conclusions

Examination of the AIE group’s result suggests that there may be an unusual increase in fEPSP slope%, possibly other than LTP. Other studies have found similar results from AIE treatments on early adolescent rats (Sabeti, 2011, Sabeti and Gruol, 2008). This appears to share characteristics with the slow transient raise in EPSP slope% that they describe. After induction of LTP by HFS an increase in EPSP slope% is expected to be found, however it stabilises quickly and does not continue to increase.

Due to this interesting result further investigation of this slow increasing form of LTP was justified. As this could by hypothesised as two separate mechanisms, there is the chance that the AIE treated effect may be HFS independent. Therefore by repeating the basic
design of experiment 4.2, but omitting the induction of traditional LTP by HFS it would be possible to investigate whether increases in fEPSP slope% are still evident in the AIE treated group, supporting the hypothesis of a separate underlying mechanism.
Figure 4.3. Experiment 2: The investigation of AIE on hippocampal LTP. Hippocampal slices of AIE treated C57BL/6J mice. Left: The post-HFS fEPSP slope% change. The control group show a 20 - 30% increase on baseline. The Ethanol group show increasingly higher values post-tetanus. Although both groups show EPSP slope% above the threshold of 120% the ethanol group couldn't quite achieve statistically significant increase over the controls (F1, 17 = 3.003, p > 0.05). Right: Representative images of EPSP spikes for, a) control pre- and post- HFS and b) AIE pre- and post- HFS.
Figure 4.4. A diagram explaining the expected and observed results from the investigation of AIE on hippocampal LTP. Normal fEPSP responses are averaged at 100%, then when LTP is induced, typically by HFS and increase in fEPSP slope% can be seen, this is traditional LTP. If LTP was not induced the fEPSP slope% should remain the same as baseline. However, in the AIE treated group there appears to be two forms of LTP occurring, one the traditional form, the other a slow increasing form that establishes itself over the recording period.
4.3.3 Experiment 3: Investigation of AIE treatments without HFS

This experiment investigated whether an increase in fEPSP slope% seen by the AIE group in experiment 2 was a product of LTP induction alone or whether it was caused by an independent mechanism (Fig 4.4). The design was identical to that of experiment 4.2 apart from the omission of the HFS step.

4.3.3.1 Experiment 3: Specific Methods

This experiment comprised of 2 groups an AIE group (N = 3) and a Control group (N = 4). Animals were sacrificed and hippocampal slices prepared on the 7th day after the end of AIE treatment. However in the experiment there was no HFS to induce LTP.

4.3.3.2 Experiment 3: Results

The results of this experiments show that even in the absence of HFS, the AIE group display a slow increase in fEPSP slope% as the experiment proceeds. At the duration equivalent to the LTP60 measure in experiment 2, the AIE group appear to show a greater than baseline fEPSP slope%, although the statistics are subject to low populations (p = 0.071). The Control group shows no signs of an increase in synaptic efficiency in the absence of HFS, and does not deviate from its baseline value for the entire duration of the experiment. A repeated measures ANOVA revealed a group by time period interaction trend (F2, 5 = 3.193, p = 0.054), suggesting that even in the absence of a HFS the AIE group is undergoing a slow increase in synaptic efficiency (Fig 4.5).

4.3.3.3 Experiment 3: Interim Conclusions

These results suggest that AIE treatment is causing a slow transient increase in fEPSP slope% that has also been found in other studies (Sabeti, 2011, Sabeti and Gruol, 2008). Various studies have shown that when ethanol acts on LTP and creates unusual effects that it is often mediated by the NMDA receptor (Sabeti, 2011, Sabeti and Gruol, 2008, Izumi et al., 2007, Tokuda et al., 2011, Tokuda et al., 2007). Therefore, there is good reason to investigate the role of the NMDA receptor in this transient increase in fEPSP slope% seen in AIE treated mice.
Figure 4.5 Experiment 3: Investigation of AIE treatments without HFS. Left: The control group maintained their baseline line levels throughout the recording duration, whereas the AIE group show a slow transient increase trend in fEPSP slope% (t = 3.544, df = 2, p = 0.071) (group by time period interaction, F_{2, 5} = 3.193, p = 0.054). Right: Representative images of the early and late periods of the recordings for, a) the control group, and b) the AIE treated group.
4.3.4 Experiment 4: Investigation of the AIE-induced LTP using MK-801

To investigate for the role of the NMDA receptor in both traditional and the slow increasing transient form of LTP, the NMDA receptor antagonist MK-801 was used. Pharmacological experiments have shown that LTP induced by HFS is NMDA dependent (Collingridge et al., 1983a, Desmond et al., 1991). For instance 10µM of the anticonvulsant (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), has been shown to inhibit the induction of LTP in hippocampal slices (Coan et al., 1987). Another reason that MK-801 was chosen was that rather than any other NMDA receptor antagonist such as APV is that, once APV is washed out its effects on NMDA receptors are removed (Tokuda et al., 2007, Tokuda et al., 2013), and due to the fast flowing, limited contact perfusion this would have needed very substantial amounts of APV, whereas MK-801 continues to antagonise NMDA receptors even after wash out. As a result even a limited dose would maintain antagonism of the NMDA receptors throughout the experiment (Huettner and Bean, 1988, Reynolds and Miller, 1988).

4.3.4.1 Experiment 4: Specific Methods

This experiment consisted of 2 groups, an AIE group (N = 6) and a saline treated Control group (N = 6). Animals were sacrificed and hippocampal slices prepared on the 7th day after the end of treatment. Additionally, during recording, 10µM MK-801 in aCSF was applied for 20 minutes before the induction of LTP by HFS. Once HFS occurred the aCSF was perfused for the remainder of the experiment.

4.3.4.2 Experiment 4: Results

The results of this experiment show that 10µM MK-801 applied prior to and throughout HFS, blocked all increases in EPSP slope% associated with the AIE treatment (p > 0.5) (Fig 4.6). Neither traditional LTP nor the slow increasing transient LTP were noticeable. Concluding that the slow increasing transient LTP created by AIE treatment is NMDA receptor-dependent.
4.3.4.3 Experiment 4: Interim Conclusions

By applying the NMDA antagonist MK-801 it was revealed that the AIE treatment generated HFS-independent LTP was abolished. Therefore it can be concluded that this mechanism is NMDA dependent for its expression.
Figure 4.6. Experiment 4: Investigation of the AIE-induced LTP using MK-801. 10µM MK-801 was added to the aCSF solution 20 minutes before HFS to block NMDA-dependent LTP. Left: Both the Control and AIE group did not show any increase in fEPSP slope% after the application of MK-801 (F1, 11 = 0.036, p > 0.5). Right: Representative images of fEPSP spikes pre- and post-HFS in, (a) Control and (b) AIE groups.
4.4 Discussion

4.4.1 The effects of various acute ethanol protocols on adolescent hippocampal LTP

It has been frequently reported that a range of ethanol concentrations can block hippocampal LTP in rodents (Morrisett and Swartzwelder, 1993, Roberto et al., 2002, Schummers et al., 1997, Tokuda et al., 2007, Izumi et al., 2007, Sinclair and Lo, 1986). These experiments replicate such findings and show that when a 60mM ethanol solution was applied to the hippocampal slice, the concentration quickly rose to 60mM (see Experiment 1: Acute ethanol, Burst and Off-On groups), and the induction of LTP by HFS was inhibited and the fEPSP’s slope% remained constant. This further supports that rapid elevations in ethanol concentration inhibit LTP in hippocampal slices (Morrisett and Swartzwelder, 1993, Roberto et al., 2002, Schummers et al., 1997, Tokuda et al., 2007, Izumi et al., 2007, Sinclair and Lo, 1986). Moreover, this inhibition of LTP occurs when ethanol is both present or absent at the moment of HFS and beyond, similar to the effect seen by Tokuda et al (Tokuda et al., 2007).

This finding is relatively well characterised. Acute ethanol’s inhibition of LTP is thought to be due to ethanol’s interaction with NMDA receptors (Schummers et al., 1997, Tokuda et al., 2007), however most hypotheses that this is mediated via ethanol’s excitation of the inhibitory GABA system (Sanna et al., 1993, White et al., 2000b, Izumi et al., 2007). If ethanol directly inhibits NMDA receptors, or indirectly via GABA mediated inhibition, the result is a reduction in Ca\(^{2+}\) influx for the use in LTP (Collingridge et al., 1983b, Collingridge et al., 1983a).

Interestingly if the ethanol concentration was increased slowly from 0mM to 60mM in 15mM steps then reduced slowly back to 0mM, LTP could be induced by HFS (Pyramid group from experiment 1). This result was similar that reported by Tokuda (Tokuda et al., 2007), where they showed that a slow increase in ethanol concentration was necessary to be able to induce LTP. However the Ramp group the ethanol concentration was also increased slowly to 60mM but then maintained, in this example LTP was not induced. Yet in Tokuda’s experiments a similar ethanol protocol was able to induce LTP even in the presence of 60mM ethanol. The burst group was treated for 30 minutes at 60mM ethanol during the baseline period, and the pyramid group was treated with 60mM ethanol.
ethanol for 20 minutes during the baseline period and at similar time points (the pyramid had 5 minutes either side of the 20 minutes at 45mM, so they are relatively comparable). Despite this, the two groups produced different results. Although the total ethanol duration/mM was close between groups, the burst group did not show LTP unlike the pyramid group. HFS in the burst group occurred long after ethanol had been washed out, therefore ethanol would not be expected to remain locally and be actively inhibiting LTP. Yet it is clear that during the ethanol period some changes occurred that inhibited the induction of LTP by HFS. This suggests that it is not just the presence of a high concentration of ethanol at the time of HFS, but rather that ethanol is having an effect upstream of the event.

### 4.4.2 Ethanol-tolerant LTP

The type of LTP that has been noticed in the pyramid group has been generally termed ‘ethanol-tolerant LTP’ (Tokuda et al., 2007, Talani et al., 2011, Sabeti, 2011). Ethanol—tolerant LTP becomes induced after a slow rise in ethanol concentration prior to the induction of LTP. This process allows for the induction of LTP to be formed independent of NMDA receptors (Tokuda et al., 2007, Izumi et al., 2015, Zorumski et al., 2014, Izumi et al., 2008, Tokuda et al., 2013, Tokuda et al., 2010, Tokuda et al., 2011). How this form of LTP develops is currently unknown. Therefore in the Pyramid group perhaps the total duration at which ethanol was at 60mM may have inhibited the development of an acute ethanol tolerance (Tokuda et al., 2007, Sabeti and Gruol, 2008), and that the slow ‘ramping’ ethanol concentration in the Ramp and Pyramid groups was not slow enough to establish the ethanol-tolerant form of LTP evident in other examples (Tokuda et al., 2011, Sabeti, 2011, Sabeti and Gruol, 2008, Izumi et al., 2008, Fujii et al., 2008, Tokuda et al., 2007). Although the Pyramid group did appear to show ethanol-tolerant LTP it could have been that the slower ramping and, ultimately the removal of ethanol was sufficient enough to be able to induce LTP.

Interestingly acute ethanol (60mM, 15 min) has been shown to increase, NMDA and AMPA and GABA receptor subunits and PSD95 as well as changes in presynaptic protein expression (Ramachandran et al., 2015). These changes can occur very rapidly and add to the complicated story of how ethanol could be inhibiting LTP. However, this could also be the mechanism by which ethanol-tolerant LTP mechanisms are formed.
4.4.3 Ethanol may stimulate allopregnanolone to induce ethanol-tolerant LTP

Another possibility that has received much attention is that ethanol also stimulates the synthesis of the GABA-A receptor enhancing neurosteroid, allopregnanolone (Izumi et al., 2007). Allopregnanolone has been suggested to modulate the physiological and behavioural effects of ethanol (Matthews et al., 2002). In the hippocampus, pyramidal neurons are immunopositive for 5-alpha-reductase neurosteroids, such as allopregnanolone (Tokuda et al., 2011, Saalmann et al., 2007), and 5-alpha-reductase inhibitors block the inhibiting effects of ethanol on LTP (Izumi et al., 2007, Ramachandran et al., 2015). Therefore, one possible explanation for ethanol-tolerant LTP is a mechanism via allopregnanolone. Although this theory fits well, it doesn’t rule out that ethanol could be modulating this effect by many other mechanisms. Moreover, little attention has been paid in these studies to the different effects seen if ethanol application is applied in a slower, more realistic manner that is likely to better mimic in-vivo changes during voluntary ethanol consumption in humans.

4.4.4 Other NMDA-independent forms of LTP

There have been other cases where traditional NMDA receptor-dependent LTP can still occur by using another mechanism or pathway, typically also leading to the influx of calcium into the neuron. There is behavioural evidence that shows that GluR_A deficient mice, tested in a spatial learning water maze, overcame the usual dependence of NMDA receptors to the point where NMDA antagonists had no effect of the results (Bannerman et al., 1995, Saucier and Cain, 1995, Saucier et al., 1996, Bannerman et al., 2014). Calcium is a critical step in any form of plasticity, it signals for many of the changes to occur in the development of LTP and plasticity (Byth, 2014). Therefore calcium must be recruited. In another study ethanol-tolerant LTP could not be wholly blocked by NMDA receptor and VDCC antagonists, however it can be blocked thapsigargin (internal store calcium inhibitor/chelator) which also blocks traditional LTP (Harvey and Collingridge, 1992, Tokuda et al., 2007).
4.4.5 The effects of AIE treatment on LTP

The physiological investigation of the AIE treatment (*experiment 2: The investigation of AIE treatment on hippocampal LTP*) found LTP in hippocampal slices from both the AIE and control groups when tested in adulthood. Interestingly, the amplitude of the LTP in the AIE treated group showed a slow persistent transient increase that was not evident in the Control group. This finding is supported by experiments from AIE treated rats where, in adulthood they too showed a slow transient increase in fEPSP slope% (Sabeti and Gruol, 2008). However, they found decreased LTP in when AIE was performed in late adolescence (PND 45 – 60) and an increase in LTP when AIE was performed during early adolescence (PND 30 – 45). Suggesting that even during adolescence there are still age-dependent differences (Sabeti and Gruol, 2008).

Experiment 3 showed that even in the absence of the induction of LTP by HFS, AIE treatment resulted in an LTP-like enhancement of EPSP slope% with no change from baseline in the control group. The magnitude of the slow transient fEPSP slope% enhancement is smaller than if a HFS was used (as it was in *experiment 2*). This suggested that these are two separate mechanisms, one, a traditional HFS induced NMDA-dependent LTP, and the other developed through AIE or similar treatments and is a HFS-independent form of synaptic enhancement. Although this was a pilot experiment, with limited N numbers, it suggested that the AIE treatment had some effect on the way the hippocampus responded in adulthood.

The higher levels of LTP seen in the AIE group in experiments 2 and 3 can be characterised by a slow persistent increase in the EPSP slope% away from control values. This may be a product of two separate LTP mechanisms which when co-expressed, result in a higher overall LTP. This could occur in a number of ways, it may be that this shares similarities with the NMDA receptor-independent slow rising LTP seen in the Sabeti studies (Sabeti, 2011, Sabeti and Gruol, 2008), if this were the case however then the AIE treated mice should still show an slow increasing LTP even in the presence of an NMDA receptor antagonist. However, in this study when the NMDA antagonist MK-801 was applied 20 minutes before HFS both the traditional LTP and the slow increasing LTP were blocked. So therefore this mechanism could not be the same as the mechanism characterised in Sabeti’s work and other possible mechanisms should be reviewed.
4.4.6 AIE may inhibit neurogenesis and cause cell death

Some studies have reported that chronic ethanol treatments result in a loss (15-20%) of pyramidal neurons (Walker et al., 1980, Obernier et al., 2002a), this may alter the way in which the remaining neurons function (Rogers and Hunter, 1992). It should be noted however, chronic treatment is much more severe than the AIE treatment that have been used in this thesis (Rogers and Hunter, 1992). However, others have reported that the chronic treatments used in these examples were not possible in humans, and that in humans, chronic drinking does not lead to hippocampal cell death (Harding et al., 1997, Obernier et al., 2002a). If the AIE treated animals in these experiments had developed hippocampal neuronal death, this could lead to instability within the hippocampal circuits, increased susceptibility to insult, where slice preparation could result in less viable slices than control treated animals. As a result the increase in LTP could be attributed to the poor health and viability of the slices. More recent work has suggested that this AIE and similar treatments are not necessarily causing hippocampal cell loss, but they could be causing a reduction in hippocampal neurogenesis (Crews et al., 2006, Crews et al., 2004).

4.4.7 The effects that ethanol and AIE have on GABA-mediated recurrent inhibition

Another possibility is that interneurons involved in the recurrent circuitry of the hippocampus are impacted by AIE treatments, in particular the CA3 – CA3 recurrent connections. A decrease in / or inhibition of, primarily, CA3 recurrent projections would result in an increase in seizure activity within CA3, and could result in increased signalling to its projections, including the CA1. Studies have indicated that ethanol treatments reduce recurrent inhibition (Abraham et al., 1981, Rogers and Hunter, 1992), and therefore could result in increased seizure activity or spontaneous spiking, and possibly altering projections towards CA1. However, it is difficult to distinguish between whether this was a mechanistic alteration in a fully functioning network, or whether this is due to cell loss, in particular interneuron cell loss or inhibition of neurogenesis (which many studies appear to ignore) which make up part of the recurrent inhibitory process. This may account for the slow increasing LTP seen as a result of AIE. If this were the case then the process is in some way NMDA receptor dependent, observed by application with MK-801 inhibiting its increase. The recurrent connections in the hippocampus are...
predominately GABA mediated. For instance, if hippocampal slices are exposed to the GABA\textsubscript{A} receptor antagonist bicuculline, the ethanol treatment modification of LTP is no longer observed (Peris et al., 1997), suggesting treatments like the AIE may be persistently modifying the GABAergic systems in the hippocampus.

Recurrent inhibition could also play a role in the LTP observed in the pyramid group of the acute treated hippocampal slices in experiment 1. It is possible that the slow increases in ethanol concentration are, producing a disinhibition of the GABA mediated inhibition. However, in a similar experiment it was suggested this could not be the case because it was found to not inhibit pop-spike paired pulse depression, therefore unlikely to account for the increase in LTP (Tokuda et al., 2007). Moreover, if acute ethanol was inhibiting GABA mediated recurrent inhibition, then it would be expected to increase levels of LTP in all ethanol treated slices, and this is not the case.

4.4.8 AIE may upregulate NMDA receptors

The upregulation of NMDA receptors is seen in human alcoholics (Julien, 2011), this may function on an ethanol use-dependent curve, where severe alcoholism produces greater numbers (Nagy, 2008). When humans who have suffered from alcoholism abstain, and go into withdrawal, excessive glutamatergic excitation can result in seizures and can be deadly (Nagy, 2008, Hopf et al., 2007, Moonat et al., 2010). The AIE treatments could have caused for an increase in NMDA receptor expression, albeit not as severe as in alcoholism. Therefore in the slice preparation this could have resulted in the increase in EPSP's due to a ‘seizure’ like activity.

4.4.9 The possible dehydration effect of ethanol on cells

Ethanol and other larger-chain alcohols are known to be able to dehydrate cells, including neurons (Le Meur et al., 2012). However, the concentrations needed to produce this effect are extreme and therefore it is unlikely to occur in the blood or CSF of mammals (Pascual et al., 2007, Pascual et al., 2009). This experiment perfused an aCSF fluid containing various ethanol concentrations (0 – 60mM), that are comparable to concentrations found in the CSF of the mammalian brain (Sabeti and Gruol, 2008, Tokuda et al., 2011, Tokuda et al., 2007, Zorumski et al., 2014). There is limited
acknowledgement of this effect of ethanol in the literature regarding hippocampal slice electrophysiology, at least at biologically relevant ethanol levels, this could be a general oversight, or perhaps a general consensus that ethanol is not having a significant effect due to its relatively low concentration. Nonetheless, there is some evidence suggesting that even very high levels of ethanol (150 – 200 mM)(a fatal concentration in rats) had no impact on membrane potential or input resistance, spike shape or EPSP in the vast majority of CA1 and CA3 neurons (Siggins et al., 1987).

4.4.10 The role of ethanol’s secondary metabolites on LTP

More recent evidence suggests that ethanol’s secondary metabolites, mainly acetaldehyde that are generated locally within the hippocampus could be a cause of synaptic dysfunction and may lead to abnormalities in LTP (Tokuda et al., 2013). The implication of acetaldehyde in the damage caused by ethanol intoxication is increasing in all areas of alcohol research (Tokuda et al., 2013, Tambour et al., 2005, Quertemont and Grant, 2002).

4.4.11 Conclusions

In conclusion, experiment 1 showed that 60mM ethanol inhibited LTP when present at HFS. It also inhibited LTP when a 60mM ethanol solution was quickly applied to the hippocampal slice for 30 minutes, then washed out 20 minutes before HFS. However, if the ethanol concentration is slowly raised and then slowly reduced before complete washout, LTP can be induced. There is speculation as to what allows for this ethanol-tolerant form of LTP, with a NMDA-dependent mechanism (Tokuda et al., 2007, Sabeti and Gruol, 2008) and allopregnanolone (Izumi et al., 2007, Tokuda et al., 2011, Izumi et al., 2015) being suggested among others as possible theories.

In experiment 2, the effects of AIE were investigated using hippocampal LTP. AIE treated slices showed a slow increasing transient increase in fEPSP slope% over time. In experiment 3 this was shown to be evident in AIE treated slices even in the absence of HFS and traditional NMDA-dependent LTP. Experiment 4 showed that this separate LTP mechanism was also NMDA-dependent, by applying MK-801 to the solution prior to HFS. Similar findings have been reported (Sabeti et al., 2007, Sabeti and Gruol, 2008, Sabeti, 2011), yet exactly what the underlying mechanisms are is unknown. The discussion
highlights several possible explanations for this novel effect including; that AIE could result in an inhibition of neurogenesis, may deregulate the GABA-recurrent inhibition in the CA3 network, possibly result in an increase in NMDA receptors which may result in seizure-like activity.

Further studies will need to be performed to understand and piece apart the complexity of how ethanol is affecting LTP, what mechanisms can be compensated for, and exactly how the already numerous (and counting) interactions ethanol appears to have on hippocampal neurons, interact with each other. Moreover, AIE treatments appear to consistently bring up similar and quite profound results. Careful investigation should be made into how these results may be affecting AIE treated behavioural studies. Also, an assessment should be able to see if human adolescents are also susceptible to these noticeable effects.

Despite the specific effects ethanol has with spatial memory and hippocampal LTP, ethanol has varied effects on behaviour and cognition, and affects so many other neural regions, some also associated with memory (Zorumski et al., 2014), such as the striatum which play a key role in instrumental learning (Lovinger, 2010). Moreover, striatal LTP is highly sensitive to ethanol and completely blocks LTP at 10mM (Zorumski et al., 2014). Therefore it is important to also consider the effects of ethanol and binge-like ethanol treatments on other testable forms of memory, such as instrumental conditioning.
Chapter 5 – The effects of ethanol treatment on operant conditioning behaviour in C57BL/6J mice

In humans, adolescent binge drinking has been known to result in a number of neural abnormalities (Spear, 2015, Vetreno et al., 2014, White, 2003, White and Wallet, 2000). Many studies have overwhelmingly indicated that use of ethanol, either in acute, chronic or binge treatments has the ability to negatively affect many and varied types of memory (Hartley et al., 2004, Tapert et al., 2004b, Weissenborn and Duka, 2003, Townshend and Duka, 2005). Ethanol has also been shown by C-fos labelling (an indirect marker of neuronal activity) to increase activity in several parts of the extended amygdala including; the Nucleus Accumbens shell, medial part of the central nucleus of the amygdala, and lateral bed nucleus of the *stria terminalis* (Leriche et al., 2008), striatal interneurons (Blomeley et al., 2011), as well as infralimbic and prelimbic sections of the mPFC (Moselhy et al., 2001). These areas are implicated in operant conditioning tasks (Baldwin et al., 2002b, Andrzejewski et al., 2013, McKee et al., 2010, Del Arco and Mora, 2008). Therefore by using the same ethanol treatment used in the previous two chapters it is possible to expand the investigation of how ethanol effects memory into a simple form of learning.

This chapter will introduce how ethanol affects operant conditioning and the consolidation/reconsolidation mechanisms involved. It will also give examples of how ethanol in certain examples can facilitate memory, as well as outlining its effects on conditioned taste aversions. Two experiments are then described: the first investigates the effects of ethanol on an appetitive instrument conditioning task and the second investigates the potential of ethanol, at the doses used in the first experiment, to induce a conditioned taste aversion.
5.1 Introduction

Operant conditioning chambers were initially developed using rats, primates and pigeons (Skinner, 1938). This was adopted and later development included using the C57BL/6J mouse, among many rodent strains. C57BL/6J mice are able to acquire and maintain lever pressing for reward in operant chambers (Kelley and Middaugh, 1996, Griffin and Middaugh, 2003) and many paradigms initially developed in other species have successfully adapted to the mouse.

It has been established that the Nucleus Accumbens (NAc) and corticostriatal systems are necessary for the acquisition and initial consolidation of a lever-pressing task with an appetitive reward (Hernandez et al., 2002, Hernandez et al., 2006). Dopamine within the NAc has been identified as a key component for operant conditioning, and is necessary for learning to lever press for a reward (Aberman et al., 1998, Salamone and Correa, 2012, Cardinal and Cheung, 2005). Once conditioned, and the memory is formed, no further protein synthesis in the NAc is necessary for the further consolidation or continued maintenance of the memory trace (Hernandez and Kelley, 2004). There is also reason to suggest the involvement of the hippocampus in instrumental conditioning as dorsal hippocampal lesions disrupt performance in instrumental conditioning (Corbit and Balleine, 2000). Other regions have been suggested in the maintenance and reconsolidation of operant behaviours. Regions like the amygdala (Baldwin et al., 2002a, Baldwin et al., 2002b, Andrzejewski et al., 2013), medial prefrontal cortex (mPFC) (Baldwin et al., 2002b, Andrzejewski et al., 2013), dorsal medial striatum (McKee et al., 2010, Andrzejewski et al., 2013) and anterior cingulate gyrus (McKee et al., 2010, Andrzejewski et al., 2013). Additionally, it has been shown that coincident activation of dopamine D1 and glutamate NMDA receptors within the mPFC is necessary for operant conditioning to occur (Baldwin et al., 2002b, Castner and Williams, 2007, Del Arco and Mora, 2008). More recent work has suggested that NMDA receptors are required during learning in the chamber but not after in a distributed network of neural regions (Andrzejewski et al., 2013). Interestingly, the temporal lobe and prefrontal cortex which contain these neural regions generally mature later than other brain regions, which could implicate them to damage from late adolescent binge drinking (Gogtay et al., 2004).
5.1.1 The effects of ethanol on the operant performance and the neuronal regions involved in operant conditioning

Acute ethanol also been shown by in vivo microdialysis to increase the levels of dopamine in the NAc (Yoshimoto et al., 1992). Acute or repeated ethanol (1g/kg i.p) increased NAc dopamine levels in adolescent rats, with repeated ethanol producing a leftward shift in peak dopamine levels (Philpot and Kirstein, 1998). Moreover, if treated with ethanol but given a saline injection prior to dopamine sampling, NAc dopamine levels still increase (Philpot and Kirstein, 1998), suggesting that dopamine levels may increase due to a prediction of ethanol, or possible the technique involved with its administration (Maldonado-Devincci et al., 2010). Repeated ethanol injections in adolescent Swiss mice resulted in an increase in extracellular glutamate levels in the NAc (Carrara-Nascimento et al., 2011). Interestingly, AIE treated animals show higher levels of basal dopamine than adults (Philpot and Kirstein, 2004). Also, NMDA receptor-mediated processes in the mPFC have been shown to be particularly sensitive to ethanol (Weitlauf and Woodward, 2008, Leriche et al., 2008).

The majority of ethanol studies that involve operant chambers and lever-pressing are investigating in ethanol self-administration and the rewarding and addictive properties of ethanol. However, there is some limited work exploring the effects of ethanol on the ability to learn and perform operant tasks. A study investigating the effects of ethanol on operant reaction time, showed that when male Wistar rats were treated with 1g/kg ethanol (i.p.) 15 minutes before testing, they experienced a significant disruption in performance (Koob et al., 1988). Additionally, in my own pilot study I found that mice injected with 2 g/kg ethanol even 30 minutes before task initiation, were unable to learn or perform the task in most cases (results not published). In another study male Wistar rats were trained that a lever press of 5s or longer after the previous one resulted in reward (a differential reinforcement of low rate schedule). After they were fully trained, testing began using i.p. ethanol injections 15 minutes before beginning the task, testing after acute ethanol injections resulted in a suppression of responding and a reduced ability to perform the task (Woudenberg and Slangen, 1988). Another study showed that 1g/kg ethanol decreased the rate of lever-pressing in rats trained on a fixed-ratio-10 schedule for water reinforcement. This was found to be dose-dependent and 0.56g/kg and 0.3g/kg doses did not reduce lever pressing rates (Jarbe and Hiltunen, 1988). This effect has been replicated in male C57BL/6J mice, albeit at much higher concentrations. C57BL/6J mice were trained on a fixed-ratio 20 reinforcement schedule for 7 days. For
testing they received ethanol (2.5g - 3g/kg) injections (i.p.) 5 minutes prior to testing. It was shown that the ethanol injections caused motor deficits and inhibition of operant behaviour (Middaugh et al., 1992).

Binge-like ethanol treatments during adolescence have reported early adolescent increases in basal NAc dopamine concentration (Pascual et al., 2009, Philpot et al., 2009). Although it is not known whether this change is temporary or persists into adulthood (Badanich et al., 2007, Sahr et al., 2004). Interestingly, an AIE treatment (significantly longer then the model used in this thesis) administered during adolescence reduced ethanol-evoked dopamine release in the mesolimbic dopamine system in adulthood (Zandy et al., 2015). However, this treatment was performed during early adolescence, and research has shown that ethanol affects adolescent periods differently (Spear, 2015, Sabeti and Gruol, 2008). However, the reduction in ethanol-evoked dopamine release could be a function of an increase baseline, such that the total dopamine in AIE and controls would be the same.

5.1.2 Ethanol's effects on consolidation and reconsolidation in mammals

After training occurs in a novel form of learning the memory ‘trace’ is subjected to protein-dependent alterations that consolidate the memory for subsequent use (Nader and Einarsson, 2010, Kemenes et al., 2006a). In these planned experiments, reconsolidation would occur once the trial was repeated. The previously consolidated memory is retrieved and further reconsolidation is required to bring the memory ‘trace’ back to its consolidated state (Nader and Einarsson, 2010, Hernandez and Kelley, 2004, Kemenes et al., 2006a). Interruption or augmentation of these processes can lead to deficits in the memory ‘trace’ (Nader and Einarsson, 2010). Various experiments have directly or indirectly explored ethanol’s effect on consolidation and reconsolidation mechanisms by performing post-trial ethanol injections. For example, male CD1 mice that were injected (i.p) with 1 or 2g/kg ethanol post-trial showed impaired retention in a single trial avoidance task (Aversano et al., 2002). It was also observed that CD1 mice, when tested in a single-trial passive avoidance task with post-trial injections of ethanol (1g/kg and 2g/kg) impaired performance (Castellano and Pavone, 1988, Castellano and Populin, 1990). In another study, post-session (3 minutes or 24 minutes) administration of 1.5g/kg ethanol in C57BL/6J mice did not affect extinction of contextual fear conditioning. However, administration of 3g/kg 24 minutes after did impair extinction (Lattal, 2007).
5.1.3 Post-learning ethanol use can facilitate memory

A study in humans has shown that alcohol can facilitate memory for material that has been recently learnt, whilst impairing subsequent material (Knowles and Duka, 2004), suggesting that post-trial injection of ethanol could improve some memories. Other studies have shown that if animals predicted they would receive ethanol, the dopamine levels in the NAc would increase (Philpot and Kirstein, 1998, Maldonado-Devincci et al., 2010). It is possible that increases in NAc dopamine would facilitate performance and increase lever pressing in an appetitive learning task (Maldonado-Devincci et al., 2010).

5.1.4 Ethanol and conditioned taste aversion

A pilot study revealed that C57BL/6J mice injected with 2g/kg ethanol 30 minutes or immediately before being placed into the operant chamber did not acquire lever pressing for a food reward. Using post-trial injections of ethanol is the closest time window that would not disrupt acquisition of the task, and yet still be able to perform am ethanol treatment during the training period. This however could have its own issues, the appetitive reward may become aversively conditioned to nausea of other dysphoric effects induced by the ethanol injection and may generate in a conditioned taste aversion (CTA) to the reward. Whilst other strains of mice (e.g. DBA/2J) have been shown to develop a CTA following administration of 2g/kg ethanol, C57BL/6J did not (Risinger and Cunningham, 1995, Broadbent et al., 2002). Moreover on a study of 15 different inbred mice strains, C57BL/6J mice were shown to have the lowest reduction in ethanol intake after 2g/Kg, and the second lowest reduction in intake after 4g/Kg (Broadbent et al., 2002).

5.1.5 Aims and Hypotheses

The two main aims of these experiments reported in this chapter are:

1. To investigate whether a repeated treatment of post-trial injections of ethanol, rather than a single injection could impair performance in an operant task.
2. To critically assess whether post-trial injections of ethanol can induce a CTA effect to responding for reward.
5.2 Methods

5.2.1 Operant learning task

Male C57BL/6J mice were housed individually in standard cages and housing conditions. To increase satiation and aid learning food was restricted to two pellets cage/day, one week before the start of the experiment to increase satiation. All subjects had their weight maintained at 85% free-feeding body weight for the entire experiment by adjusting their diet accordingly, but never below one pellet per day.

Two operant chambers were used between phases 1 and 2. These have already been previously described in the general methods section. The following describes the procedure followed in both operant chambers.

5.2.1.1 Habituation

A single habituation trial was conducted in phase 1 and phase 2 in order to allow the subject to familiarise itself and associate the operant chamber with food reward (24 hr before trial 1). Procedure was as follows: the subject was placed into an unlit operant chamber, 30s later the house light comes on and a food reward is dispensed into the food magazine. Food rewards continued to be dispensed every following minute, resulting in a total of 29 pellets during the 30 minute habituation trial.

5.2.1.2 Training trials

Each training trial started with the subject being placed into the operant chamber which was then sealed and an internal home cage light comes on. 30s into the trial both levers were presented to the subject. Upon each successful lever press a single food reward was dispensed into the food magazine. Following a lever press (correct or incorrect) both levers were withdrawn for 15s, before being presented again. Each trial lasted for 30 minutes, and at the end of the trial both levers were withdrawn and the house light turned off, the subject was then removed. Immediately after the trial ended the subject received an appropriate injection and was then placed back into their home cage. The amount of lever presses and head entries were recorded for analysis. A lever press involved the
mouse pressing the lever sufficiently to give a reward. A head entry involved the mouse placing its head into/near the region where the reward was presented.

### 5.2.2 Conditioned taste aversion

All subjects were first adjusted to a limited water schedule by only allowing water access for four hours a day (10:00 – 14:00), starting four days prior to week one and then continued throughout the experiment. The CTA experiment lasted 2 weeks, with each daily step occurring in each week.

**Baseline water test:** Over the first two days the weight of water consumed was then determined by weighing the water bottles after 30 minutes of access and the mean of these score was considered the baseline for each animal. After the 30 minute baseline period, a further 3.5 hours water access was given an hour after the baseline recording was taken, to complete their four hour access to water.

**Post-trial ethanol treatment day:** On day 3, all animals received 30 minutes access to a 10% sucrose solution. Immediately after they received either a; 2g/kg or 4g/kg ethanol or saline injection (i.p. 20% v/v). The mass of sucrose solution consumed was measured. After an hour break the animals were allowed 3.5 hours access to water.

**CTA test day:** On day 4, the possibility of a CTA to sucrose was tested. All animals had 30 minutes access to a 10% sucrose solution. The mass of sucrose solution consumed was measured. After an hour break all animals had 3.5 hours access to water.

**Water test:** On day 5, all animals had access to water for 30 minutes; the mass of water the consumed was measured. The water test day, evaluates whether a CTA has been generalized to any other stimuli (drinking bottle etc.). One hour after testing all animals had access to water *ad libitum.*
5.3 Results

5.3.1 Operant Learning Task: Phase one

5.3.1.1 Specific Methods

16 male adult C57BL/6J mice formed two groups; Group ES (ethanol-saline) (N = 8), that received post-trial ethanol injections (2g/kg) in phase 1 of the experiment, and post-trial saline injections in phase 2. And Group SE (saline-ethanol) (N = 8), that received post-trial saline injections in phase 1 of the experiment and post-trial ethanol injections (2g/kg) in phase 2.

Habituation followed by 5 trials were undertaken in phase 1, on a 2-day-on 2-day-off schedule, fitting with the AIE ethanol treatment protocol. Phase 2 consisted of 8 trials (one habituation, 7 training trials) on a 2-day-on 2-day-off schedule. In total there were 12 training/testing days (Fig 5.1).
Figure 5.1. Treatment and testing protocol for the investigation of AIE on instrumental learning. There is a habituation day on the first day of both phases. Group ES received ethanol injections post-training on a 2-day-on 2-day-off basis in phase 1, group SE received saline in the same pattern. The treatment groups were switched in phase 2, now group ES is injected post-training with saline and group SE with ethanol.
5.3.1.2 Results

Numbers of lever presses and head entries into the food dispensing area were recorded during each 30 minute trial and averaged for each group for analysis (Fig 5.2). A repeated measures ANOVA revealed that post-trial injections of ethanol acted to reduce lever pressing in subsequent trials in the ES group when compared to the SE group (treatment by trial interaction, p < 0.05) (Fig 5.2). Post-hoc analysis showed that the ethanol treated group resulted in a significant difference between the two groups lever pressing activity at trial 5 (p < 0.05). The ethanol group reduced lever pressing in each subsequent trial, whereas the control group became increasingly conditioned to lever press. At trials 4 and 5 the controls reached a ceiling value, averaging around 50 lever presses within the 30 minutes trial period.

5.3.1.3 Interim Conclusions

To conclude, the ES group received ethanol injections post-trial and showed no improvement in the lever pressing as the trials progressed. Whereas the SE group received saline injections post-trial and learnt the task well. To observe whether a conditioned taste aversion developed in the ES group both groups were tested in different boxes, with different rewards and would now receive the opposite injection treatment.
Figure 5.2. The effects of ethanol on operant conditioning. Group ES received ethanol post-trial in phase 1 (Top-Left) and saline in phase 2 (Top-Right). Group SE received saline in phase 1 and ethanol in phase 2. Top-Left: Lever presses in trials where mice receive injections of ethanol or saline post trial. A repeated measure ANOVA demonstrated that post-trial injections of ethanol resulted in less lever pressing in subsequent trials (treatment by trial interaction, $F_{3.965, 19} = 4.077, p < 0.05$) and at trial 5 the groups showed a significant difference ($T_{12} = 4.405, p < 0.05$). Top-Right: The groups used in the graph in the top-left have their treatments switched. An already established lever pressing behaviour is reduced in the SE group after receiving post-trial injections of ethanol in phase 2 (Repeated measures ANOVA, $F_{2.017, 9} = 3.354, p < 0.05$). Conversely an increase in the ES group was observed in phase 2 ($F_{2.049, 9} = 10.069, p < 0.05$). Asterisk indicate significance between groups at trial 1 ($T_{12} = 3.167, p = 0.03$) and trial 4 ($T_{12} = 3.705, p < 0.003$). Bottom-left: shows the head entries for each trial of phase 2. The SE group make fewer head entries to the reward location as the trials progress when compared to the ES group (Repeated measures ANOVA, treatment by trial interaction, $F_{2.211, 12} = 4.354, p < 0.05$). Significance occurs between the groups on trial 4 onwards (trial 4: $t = 6.056, p < 0.05$, trial 5: $T_{9.657} = 5.804, p < 0.05$; trial 6: $T_{8.209} = 4.820, p < 0.05$; trial 7: $T_{9.106} = 3.567, p < 0.05$). Error bars indicate ±SEM.
5.3.2 Operant Learning Task: Phase two

5.3.2.1 Specific Methods

The treatment groups were reversed in phase 2 and this accounts for the SE group’s strong initial response in the first trial. And for the similarity between the values in trial 1 of phase 2 are those trial 5 of phase 1 (Fig 5.1). Moreover the SE group were ethanol naïve (injections were post-trial) until phase 2 trial 2.

5.3.2.2 Results

A repeated measures ANOVA revealed that post-trial injections of ethanol in the SE reduced lever pressing in animals who had previously learnt, whereas the ES group increased the number of lever presses (treatment by trial interaction, p < 0.05; SE group main effect of trial, p < 0.05; ES group main effect of trial, p < 0.05). Using post-hoc independent t-tests revealed that the groups differ at trial 4 and show a trend at trial 1 (trial 4, p < 0.05; trial 1, p = 0.08).

Analysis of head entries indicated that the SE group reduce head entries across sessions whilst controls do not (treatment by trial interaction, p < 0.05; main effect of trial, p < 0.05). Post-hoc t-tests revealed that trials 4, 5, 6 and 7 were showed significantly different head entries between the groups (independent t-tests; (trial 4, p < 0.05; trial 5, p < 0.05; trial 6, p < 0.05; trial 7, p < 0.05).

5.3.2.3 Interim Conclusions

Given the results from both phases of the operant learning task it can be concluded that post-trial injections of 2g/kg ethanol causes a subsequent reduction in lever pressing and head entries. How ethanol caused this behaviour is unknown. To evaluate the possibility that the reduction in lever pressing was due to a CTA impairing performance, an experiment using a similar dose of alcohol to that in the present experiment was conducted.
5.3.3 Conditioned Taste Aversion

This experiment investigated whether a single injection of 2g/kg or a higher dose of 4g/kg ethanol were sufficient to produce a CTA to a 10% sucrose solution.

5.3.2.1 Specific Methods

24 male adult C57BL/6J mice formed three equal sized groups; a 2g/Kg ethanol (N = 8), and a 4g/Kg ethanol (N = 8) and a saline injected control group (N = 8). All animals were housed individually in standard cages and housing conditions. A 10% sucrose solution was made daily using Tate and Lyle sugar (sucrose) and tap water. The sucrose solution was made available to the mice in the same style bottles, and in similar volumes to their standard cage water.

All subjects were first adjusted to a limited water schedule by only allowing water access for four hours a day (10:00 – 14:00), starting four days prior to week one and then continued throughout the experiment. The CTA experiment lasted 2 weeks, with each daily step occurring in each week.

**Baseline water test:** Over the first two days the weight of water consumed was then determined by weighing the water bottles after 30 minutes of access and the mean of these score was considered the baseline for each animal. After the 30 minute baseline period, a further 3.5 hours water access was given an hour after the baseline recording was taken, to complete their four hour access to water.

**Post-trial ethanol treatment day:** On day 3, all animals received 30 minutes access to a 10% sucrose solution. Immediately after they received either a; 2g/kg or 4g/kg ethanol or saline injection (i.p. 20% v/v). The mass of sucrose solution consumed was measured. After an hour break the animals were allowed 3.5 hours access to water.

**CTA test day:** On day 4, the possibility of a CTA to sucrose was tested. All animals had 30 minutes access to a 10% sucrose solution. The mass of sucrose solution consumed was measured. After an hour break all animals had 3.5 hours access to water.

**Water test:** On day 5, all animals had access to water for 30 minutes; the mass of water the consumed was measured. The water test day, evaluates whether a CTA has been
generalized to any other stimuli (drinking bottle etc.). One hour after testing all animals had access to water *ad libitum.*

5.3.2.2 Results

Week 1

The initial water baseline and sucrose pre-test values are highly comparable between all groups (**Fig 5.3**). Analysis of the test day showed that the groups consumed different amounts of sucrose solution (*p* < 0.05). Post-hoc t-tests show a difference between all three groups on the sucrose test day (Control v 2g/kg, *p* < 0.05; Control v 4g/kg, *p* < 0.05; 2g/kg v 4g/kg, *p* < 0.05).

To test for a CTA, the group’s consumption was compared between sucrose pre and post-treatment days. The control group increased their sucrose solution consumption (*p* < 0.05), the 4g/kg ethanol group reduced their sucrose solution consumption (*p* < 0.05); and the 2g/kg treated group consumed the same amount of sucrose solution and did not experience the increase in consumption that the control did. The final water test shows that all groups display similar consumption, which suggests that there has been no generalized aversion or preference in any groups.

Week 2

Week 2 explored whether the effects of week 1 persisted and whether multiple pairings of sucrose and ethanol could increase the likelihood of developing a CTA (**Fig 5.3**).

As with week 1 the baseline consumptions of water were matched between groups. The sucrose pre-treatment results show equal values between the 2g/kg and control group. However, the 4g/kg group display a significantly lower consumption of sucrose than controls (control v 4g/kg, *p* < 0.05). The sucrose test replicated the results from week 1, showing that 2g/kg ethanol, like controls, was unable to produce any significant change between the sucrose pre-injection and post-injection tests (control, *p* > 0.05; 2g/kg, *p* > 0.05). Using independent t-tests revealed that the 4g/kg group show lower consumption values than both the 2g/kg and control groups (Control v 4g/kg, *p* < 0.05; 2g/kg v 4g/kg, *p* < 0.05) but the 2g/kg did not differ from the controls (2g/kg vs control, *p* > 0.05). The
final water test day showed all groups were again equal, suggesting that there was no CTA generalisation.

**5.3.2.3 Interim Conclusions**

To conclude, the injection of 4g/kg produced a profound and significant aversion to sucrose and reduced subsequent intake. 2g/kg produced much more subtle effects and although it did not reduce the amount of liquid it consumed between pre and post injection days, the 2g/kg group did show a reduction in consumption when compared to the saline group, and therefore could be considered as a CTA.
Figure 5.3 Conditioned taste aversion to ethanol at 2g/kg and 4g/kg over 2 weeks. A baseline consumption was measured to water on the first 2 days (averaged to a score). The sucrose treatment day allows access to sucrose followed by an appropriate injection. The sucrose post-treatment test measured the consumption to sucrose 24 hr after conditioning. (Left): Week 1. Mice show equal liquid consumption to water on the baseline days and sucrose on the treatment day. The post-treatment test day showed different sucrose consumptions between the groups (F2, 23 = 35.074, p < 0.05). Saline injected controls showed higher consumption levels than 2g/kg (T10.570 = 4.072, p < 0.05), and 4g/kg (T14 = 9.592, p < 0.001). The 2g/kg treated also showed greater consumption levels than the 4g/kg (T14 = 3.882, p < 0.05). All groups show equal consumption of water on the final test suggesting that no aversion has been developed towards the drinking bottle or drinking itself. Paired t-tests between the pre and post treatment sucrose days revealed the saline group increased consumption (T7 = 8.485, df = 7, p < 0.05), and 4g/kg reduced consumption (T7 = 7.392, p < 0.05). (Right): Week 2. All groups show a similar response to baseline. The treatment day shows that the 4g/kg group continue to show a CTA to sucrose (T7.138 = 3.582, p < 0.05). The post-treatment day revealed a continued difference between the 4g/kg group and both the 2g/kg and control groups (2g/kg: T13 = 3.439, p < 0.05. Controls: t13 = 4.522, p < 0.05). The difference between the 2g/kg group and controls does not show statistical significance (T14 = 1.981, p > 0.05). Error bars are ±SEM.
5.4 Discussion

The results from this chapter illustrate that when C57BL/6J mice are trained to lever press in an operant box for an appetitive reward, post-trial injections of 2g/kg ethanol have a detrimental effect on the animal’s subsequent performance of the task, when compared with controls. In fact, post-trial ethanol injections appeared to inhibit the acquisition of the lever-pressing for reward in the ES group. Post-trial ethanol injections also resulted in less lever pressing in subsequent trials in previously well-trained animals like the SE ethanol. It is clear that post-trial injections of ethanol reduces lever pressing in subsequent trials.

5.4.1 Possible CTA effect

One possible explanation for this effect would be what was originally speculated, the generation of a CTA with ethanol acting as the US. It had been anticipated that post-trial injections of ethanol could lead to a CTA towards the appetitive reward, despite relevant literature suggesting that 2g/kg ethanol was not sufficient to produce a CTA in the C57BL/6J mouse strain (Broadbent et al., 2002). However, it was felt that conclusive first-hand evidence was needed to help evaluate this finding. However, the results from the CTA experiment are somewhat ambiguous. What was clear, was that 4g/kg certainly produced a strong CTA towards sucrose, as predicted from relevant literature (Broadbent et al., 2002). The 4g/kg group reduced subsequent sucrose consumption on the sucrose post-injection day dramatically. This effect lasted for at least two weeks and resulted in a reduction of sucrose consumption on the following day, prior to the next injection of ethanol. Although the 2g/kg dose did not significantly decrease the consumption of sucrose between pre- and post-injection days, what was revealed was that the level of sucrose consumed on the test day in the 2g/kg group was significantly lower than controls during week 1. So although the 2g/kg group is not reducing their consumption, they are not increasing consumption of sucrose, like controls which may indicate a CTA towards sucrose. Additionally, there is also some level of ambiguity over whether a sucrose solution would be expected to have a stronger or weaker salience as a CS when compared with either sugar pellets or a sweet strawberry milkshake.

Another possibility is that even if a single post-trial injections of 2g/kg ethanol does not result in a CTA towards sucrose, repeated post-trial injections, as used in the operant task reported, still may. The effect of this difference in pattern of ethanol treatment would
need further investigation to determine whether two or more consecutive post-trial injections would result in a CTA to sucrose.

If the CTA explanation for the reduction in lever pressing due to post-trial injection of ethanol is hypothetically rejected, there are other potential explanations of how such an effect could occur.

5.4.2 Possible delayed punishment

One possibility is that after the trial, the coincidence of an immediate ethanol injection may cause discomfort and could be interpreted as a punishment. However, the control mice also received injection post-trial and continued to increase/maintain performance. Even if ethanol intoxication alone was unpleasant then the mice may have associated the operant chamber with a later punishment and unwilling to participate in future trials. However, it is known that the C57BL/6J mice are an ethanol-prefering mouse, and will eagerly self-administer ethanol (Kelley and Middaugh, 1996, Risinger et al., 1998, McClearn, 1959), which may limit the “negative emotional” effects of ethanol upon them.

5.4.3 Ethanol treatment could be affecting memory consolidation and reconsolidation

In phase 1 the ES group was injected with ethanol after the first training trial, ethanol could be inhibiting the initial formation of the memory. Therefore, when these animals started phase 2 of the experiment, they responded as if this were a new task and acquired level pressing over the following four sessions. In phase 2, the ES group, who had previously learnt the task under saline, progressively reduced their lever pressing once they started receiving ethanol injections, this was not an abrupt cessation of lever pressing but rather a transient reduction in performance.

Therefore another possibility is that ethanol is interfering with either/both of consolidation or reconsolidation. This would be supported by various studies where post-trial ethanol resulted in an alteration of performance consistent with an interaction with either/both consolidation or reconsolidation (Aversano et al., 2002, Castellano and Populin, 1990, Castellano and Pavone, 1988, Lattal, 2007). These are consistent with the hypothesis that ethanol is impairing the consolidation/reconsolidation of memory. However, they are
also consistent with the hypothesis that ethanol is acting aversively on the subject, and could lead to a CTA (Cunningham et al., 1997, Bevins et al., 2000, Lattal, 2007).

The striatal regions that are involved in instrumental learning (Lovinger, 2010), are highly sensitive to ethanol and can even convert LTP into LTD if the ethanol concentration is above 50mM (Yin et al., 2007). Therefore it is possible that the animals that are treated with post-trial ethanol are inducing LTD and not LTP in the striatum, this may have some effect on the reduction in performance. However, it is not known whether ethanol would need to present throughout the experiment and not just post-trial.

5.4.4 Predicting ethanol did not improve performance

Though in humans post-trial ethanol can lead to facilitated memory (Knowles and Duka, 2004), and that on subsequent trials the mice may possibly have predicted for ethanol, which may have resulted in increased dopamine levels in the NAc (Philpot and Kirstein, 1998, Maldonado-Devincci et al., 2010). There was no evidence that post-trial injection facilitated memory.

5.4.5 Conclusions

Post-trial injections (i.p.) of 2g/Kg ethanol impaired performance of lever pressing for an appetitive reward in the operant conditioning task. A CTA experiment revealed inconclusive results where 2g/kg ethanol injection did not show a strong CTA effect when comparing the pre- and post- injection sucrose consumptions, unlike 4g/kg. However, it did not increase consumption of sucrose the in the post-injection trial, unlike the controls. Moreover, because the investigation of a possible CTA did not fully reflect the repeated injection model used in the operant conditioning task, it remains inconclusive. It is not known whether repeated injections would result in a greater CTA, and therefore further investigations should consider this possibility. However, evidence was provided suggesting other theories. One explanation is that ethanol may be affecting the memory consolidation/reconsolidation pathways. Acting to inhibit the molecular mechanisms involved. Although, most supporting evidence could also be concluded by a CTA effect.

An alternative strategy by which memory acquisition, consolidation/reconsolidation and retrieval mechanisms can be investigated precisely is by using a molluscan model of
learning and memory. The snail *Lymnaea stagnalis* is especially suitable since many of the molecular underpinnings of appetitive learning circuitry have been identified and temporally characterized and robust appetitive conditioning tasks have also been developed (Kemenes, 2013, Kemenes et al., 2011, Wan et al., 2010, Michel et al., 2008, Fulton et al., 2008). Therefore this thesis now expands into an investigation of the effects of high concentrations of ethanol on memory in the snail, *Lymnaea stagnalis*. 
Chapter 6 – The effects of ethanol on associative learning in *Lymnaea stagnalis*

The previous chapters have explored how ethanol affects learning by using highly testable forms of memory such as spatial memory and operant conditioning in the mouse. As many of the molecular mechanisms underlying acquisition and consolidation of memories are conserved between invertebrate and vertebrate animals (Kemenes, 2013), the use of suitable invertebrate model organisms can lead to the removal of much of the complexity that can overshadow results and allow for ‘simpler’ hypotheses to be set up and tested. One such model organism is the pond snail *Lymnaea stagnalis*, a long-established molluscan model to investigate the evolutionarily conserved cellular and molecular mechanisms of memory function and dysfunction (Kemenes, 2013). An important question that can be tested using *Lymnaea* is how high concentrations of ethanol effect acquisition, consolidation or retrieval of associative memory after single-trial learning and this is what I set out to achieve in Chapter 6.

This chapter initially explores key aspects of learning and memory that can be researched using *Lymnaea* as well as the key differences between *Lymnaea* and mice, and the benefits and limitations of using such a model. I will also review the literature regarding ethanol’s effects on *Lymnaea* and how it affects learning and memory in other invertebrates. Five experiments are then presented: The first experiment was performed to find a suitable ethanol concentration and post-injection interval to be used in subsequent experiments. In the next experiment, the effects of ethanol on associative memory consolidation were explored, followed by an investigation of the effects of ethanol on the retrieval of associative memory. The latter two experiments led to an investigation of whether ethanol treatment could result in state-dependent memory in *Lymnaea*. Finally, state-dependency was further investigated using drugs influencing biochemical mechanisms known to be involved in mediating the effects of alcohol on synaptic plasticity and learning and memory.

### 6.1 Introduction

Contrary to the complexity of the mouse brain, the snail, *Lymnaea stagnalis* has a much simpler central nervous system (CNS) consisting of around 20,000 neurons (Benjamin, 2008). Research in *Lymnaea*, and other molluscs, such as *Aplysia californica* and...
Hermissenda crassicornis and other invertebrates, such as Drosophila, C. elegans and the honey bee Apis mellifera has led to step changes in the understanding of how the nervous system works, especially in the fields of synaptic and non-synaptic plasticity and learning and memory (Kemenes, 2013, Benjamin et al., 2000, Elliott and Benjamin, 1989, Menzel et al., 2013).

Many of the neurons of the snail CNS have been characterised both anatomically and electrophysiologically, and individual neurons are readily identifiable between different animals, based on size, position, axonal morphology, spike firing pattern, synaptic inputs and outputs, transmitter content, and pharmacological responses. (Vavoulis et al., 2007, Kojima et al., 1997, Staras et al., 2002). These neurons form networks, much like the mammalian brain, allowing the generation of well-defined behaviours, such as feeding, respiration, whole-body withdrawal and locomotion to occur (Benjamin, 2008). Possibly the best studied of these Lymnaea networks is the feeding system (Elliott and Benjamin, 1989). The Lymnaea feeding behaviour can be conditioned by using associative learning protocols, such as classical and operant conditioning (Benjamin et al., 2000) and therefore it is possible to investigate the neuronal plasticity involved in the encoding of associate memory traces in this relatively simple circuit. Notably, Lymnaea lends itself perfectly to behavioural and electrophysiological experiments aimed at understanding plastic changes in the nervous system underlying precisely timed changes in behaviour. Its suitability as a model for analysing the above changes is due to the combination of its ability to form associative memories after a single trial and its well-characterised network of central pattern generator (CPG) and modulatory interneurons and motoneurons, many of which have large cell bodies making them amenable to electrophysiological analysis. Thus Lymnaea is considered to be an eminently tractable invertebrate model of learning and memory that allows the cellular and molecular analyses of highly conserved mechanisms of both memory function and dysfunction (Kemenes, 2013, Straub et al., 2006, Staras et al., 1999, Kemenes et al., 1997).

Although there are significant differences in the complexity of both the nervous system and the behavioural repertoire between Lymnaea and the C57BL/6J mouse, they both form NMDA receptor dependent associative memories (Mondadori et al., 1989, Benvenega and Spaulding, 1988, Wan et al., 2010) and do so using remarkably conserved biochemistry (Berlucchi and Buchtel, 2009, Kemenes et al., 2006a, Kemenes et al., 2002, Hatakeyama et al., 2006, Ribeiro et al., 2003, Ribeiro et al., 2005, Korneev et al., 2005, Michel et al., 2008, Pirger et al., 2010, Naskar et al., 2014). One of the benefits of having a relatively simple nervous system is that it allows for the investigation
of more direct questions about the effects certain substances have on learning and memory, such as ethanol. The range of behavioural response in Lymnaea is limited compared to mice; removing much of the ambiguity that comes with behavioural research in mammals. Also, mammals often have to be trained multiple times before a memory is fully consolidated ensuring that these multiple trials of learning have the same context is very difficult and in some cases impossible, this can lead to a memory being consolidated/reconsolidated under different conditions and resulting in a more complex memory trace (Nader and Einarsson, 2010, Hernandez and Kelley, 2004, Kemenes et al., 2006a). Conversely, one of the major advantages of using Lymnaea is to utilize the well-established single-trial classical conditioning paradigm (Alexander et al., 1984). This paradigm allows for the investigation of learning-induced behavioural and neuronal changes in a precisely timed manner (Kemenes, 2013, Benjamin et al., 2000, Kemenes et al., 1997). This also allows the experimenter to specifically target acquisition or different stages of consolidation without having to factor in complex drug side-effects as a result of multiple drug-treated trials. In Lymnaea it is possible to investigate the effects of ethanol on well-defined networks of neurons, or even single neurons that are known to be involved with the feeding/memory system. Moreover, it is possible to readily identify the same individual neuron between animals; in the mammalian brain this is not possible.

Not all the benefits of working with a simpler organism are purely scientific. By choosing an invertebrate model over a mammalian model one is able to reduce the dependence on mammals, complying with “the three R’s” principle of animal research (Tannenbaum and Bennett, 2015). Therefore, not only does this series of experiments investigate the effects of ethanol on plasticity, but also the possibility of the snail, or other invertebrates to replace some aspects of scientific research currently carried out using mammals.

6.1.1 Classical conditioning in Lymnaea

Classical conditioning is well-established in Lymnaea, and has been used successfully in the study of the cellular and molecular mechanisms involved in learning and memory (reviewed in (Kemenes, 2013)). Single-trial food-reward classical conditioning in Lymnaea (Alexander et al., 1984) consists of pairing a neutral chemical stimulus, amyl acetate in this case (which is also widely used in classical conditioning experiments in vertebrates (Walker et al., 1986, Pourtier and Sicard, 1990, Dorries et al., 1997, Paschall and Davis, 2002, Jones et al., 2005, Pavesi et al., 2011), with a salient unconditional stimulus (US), sucrose. After a single pairing, the snail learns that amyl acetate predicts
the US and thus becomes a conditioned stimulus (CS) (from herein even before being paired with the US, amyl acetate will be referred to as the CS). The association between the CS and US is consolidated by gene transcription and protein synthesis dependent formation of LTM (Fulton et al., 2005, Kemenes, 2013). Associate LTM in Lymnaea has been shown to last up to 21 days (Fulton et al., 2005, Alexander et al., 1984, Kemenes et al., 2002). Once a memory is acquired it undergoes consolidation, a protein synthesis dependent phase which results in a robust, retrievable memory (McGaugh, 2000, Kemenes, 2013, Fulton et al., 2005). Once a consolidated memory is later retrieved the memory trace transforms into a fragile, labile phase where further reconsolidation is needed to further strengthen the memory (Nader et al., 2000, Nader and Einarsson, 2010, Hernandez and Kelley, 2004, Kemenes et al., 2006a).

The molecular underpinnings of memory acquisition, consolidation, retrieval and reconsolidation have been well-studied and have been shown to be highly conserved between mammals and snails (Berlucchi and Buchtel, 2009, Kemenes et al., 2006a, Hatakeyama et al., 2006, Kemenes et al., 1997, Kemenes et al., 2002, Ribeiro et al., 2003, Ribeiro et al., 2005, Korneev et al., 2005, Michel et al., 2008, Pirger et al., 2010, Naskar et al., 2014). One of the most important initial findings that indicated a high homology, was the discovery of the CREB genes and proteins in Lymnaea. (Ribeiro et al., 2003, Sadamoto et al., 2004, Hatakeyama et al., 2004). This was an important discovery because of CREB’s known role in the conversion of STM to LTM. When this was tested in Lymnaea it was found that after single trial associative appetitive conditioning, CREB was phosphorylated in feeding circuit neurons (Ribeiro et al., 2003), for the first time confirming highly homologous memory processes between Lymnaea and other model organisms used in learning and memory research.

Other conserved molecular processes have also been identified, multiple protein kinases have been implicated with learning and memory in Lymnaea, including PKA, PKC and MAPK, with PKA and MAPK being crucial for the consolidation of long-term memory (Ribeiro et al., 2005, Michel et al., 2008). Calcium calmodulin kinase II (CaMKII) too was identified and was shown to be required for both acquisition and intermediate and late-phase memory consolidation, with acquisition needing both the NMDA receptor and CaMKII, but intermediate and late consolidation only needing CaMKII (Wan et al., 2010, Naskar et al., 2014).
6.1.2 Receptor homology between *Lymnaea* and mammals

Ethanol’s effect on mammals is likely to result from complex interactions with neuronal systems and receptors, notably GABA and NMDA receptors. Therefore for *Lymnaea* to be a suitable model for studying the effects of ethanol on plasticity, both the downstream molecular mechanisms and the upstream neurotransmitter receptors need to be homologous.

NMDA-like receptors were first identified electrophysiologically in *Lymnaea* in 1993 (Moroz et al., 1993). Since then two NMDA-type receptors have been cloned from the *Lymnaea* CNS, showing that the *Lymnaea* NR subunits belong to the NR1 family (Ha et al., 2006). AMPA receptors and subunits (GluR1-4, Glu5-7) were also identified, all of these receptors show strong homology between *Lymnaea* and mammals (Hutton et al., 1991, Stuhmer et al., 1996). GABA-like receptors also have been characterised and shown to be bicuculline and benzodiazepine sensitive (Zaman et al., 1992). The application of GABA to the RPeD1 cell was found to hyperpolarize and inhibit its firing (Moccia et al., 2009). Moreover, when the GABA(A) antagonist picrotoxin (PTX) was applied it was found to alleviate the inhibition, and allow the cell to fire (Moccia et al., 2009). These findings suggest that the GABA receptors in this system share the action sites for GABA and PTX with mammals.

6.1.3 Effects of ethanol on *Lymnaea* and other invertebrates

There have been no previous studies on the behavioural effects of ethanol on *Lymnaea* or other molluscs. All previous literature of ethanol on molluscs were based on measuring electrophysiological effects and therefore will be discussed in the next chapter. Given the absence of literature on the behavioural effects of ethanol on *Lymnaea*, other invertebrate models of learning and memory were reviewed for the effects of ethanol on memory.

In the fruit fly *Drosophila*, ethanol has been shown to have significant interactions with the *slo-1* gene (Scholz and Mustard, 2013, Ghezzi et al., 2012, Bettinger and Davies, 2014), which is the homolog of the mammalian KCNMA1 gene (Wang et al., 2009). This gene encodes for the α subunit of BK-type calcium-activated potassium channel (Bettinger and Davies, 2014). Loss of function of *slo-1* develops a phenotypic resistance to the locomotor impairing effects of ethanol (Scholz and Mustard, 2013, Davies et al.,
This gene is shared with mammals, and it has been shown to underlie a form of ethanol tolerance in the rat (Pietrzykowski et al., 2008). A similar result was found in Caenorhabditis elegans (C.elegans), where slo-1 mutants were found to be resistant to ethanol and slo-1 gain-of-function mutants exhibited “drunken behaviour” (Davies et al., 2003a, Crowder, 2004), and signs of behavioural intoxication at similar doses to humans and other mammals (Davies et al., 2003a). A genetic Drosophila mutant cheapdate (Cheapdate is an allele of amnesiac gene), increases the sensitivity to the effects of ethanol (Waddell et al., 2000). The amnesiac gene encodes a neuropeptide that activates the cAMP pathway. When the investigator increased the levels of cAMP or PKA the sensitivity to ethanol was removed, suggesting the involvement of cAMP in the response to ethanol in invertebrates (Moore et al., 1998). The honey bee, Apis mellifera is used in a model of learning and memory that uses a food-reward protocol similar to the one used in Lymnaea, albeit based on an odour conditioned stimulus. It was found that if they received ethanol before acquisition, the memory was impaired. Conversely, consumption of ethanol after conditioning did not affect memory (Mustard et al., 2008), suggesting that ethanol may affect acquisition but not consolidation.

6.1.4 Aims and Hypotheses

It is hypothesised that due to the high level of molecular homology that underpins learning and memory between vertebrates and invertebrates, ethanol may also show some degree of inhibition to learning and memory processes in Lymnaea. As ethanol is such as complex drug and has varied effects on receptor functioning at various concentrations, it was hypothesised that reducing the complexity of the model system and behavioural tasks could yield interesting results in answer to the question of how ethanol affects associative learning and memory.

The two main aims of the work presented in this chapter are:

1. To establish a suitable concentration of ethanol to use in Lymnaea behavioural experiments.
2. To investigate the effects of ethanol on the memory systems involved with classical conditioning in Lymnaea.
6.2 Methods

The following agents were all from Sigma and made into solution by dissolving in snail saline: GABA (80µM); Picrotoxin (400µM); NMDA (2mM); Ketamine (250µM). (Romanova et al., 1996, Woodall and Mccrohan, 2000, Moccia et al., 2009, Browning and Lukowiak, 2008, Rosenegger and Lukowiak, 2010).

6.2.1 Classical conditioning and testing

The following protocols have been used extensively in our lab and have been proven to establish strong, retrievable long-term memory, whilst also leaving open critical time-windows to allow for suitable pharmacological intervention (Wan et al., 2010, Kemenes et al., 2002, Michel et al., 2008, Naskar et al., 2014). Below is the standard behavioural training procedure. Some experiments will differ slightly and where this occurs it will be specifically stated.

Prior to any training snails were collected from our breeding facility and kept in plastic containers filled with Cu\(^{2+}\)-free water (18 - 20°C). All snails were food deprived for two whole days and three nights before training. This decreases the satiety level of the snails and increases the chance of successful, robust conditioning (Alexander et al., 1984, Audesirk et al., 1982).

All training was performed in Petri dishes containing 90ml of Cu\(^{2+}\) free water. Before testing, the snails were acclimatised for 15 minutes. Training commenced with 5ml of the CS being added to the water by syringe. 30s later, the US was then applied in the same manner. Two minutes later the snails were removed and placed back into their home tub. It was decided that the untrained or ‘naïve’ group would receive sucrose during training but that it would not be paired or explicitly un-paired with the CS. The reason for this is that these experiments are conducted over more days than typical single-trial conditioning in *Lymnaea* and pilot studies showed that the then naïve, now untrained (i.e. US only) group rasped significantly to water (results not shown). This could have been countered for by giving the naïve snails some food instead, but balancing the calorific quantities of sugar with lettuce was not possible. If the snails had received an amount of solid food, subsequent testing could have detected a reduction in consummatory rasping due to the animals being less hungry.
Testing started at least 24 hours after training, also in some cases 48 hours post-training, by placing the snails into a Petri dish containing 90ml of copper-free water for 15 minutes. First, a baseline response was measured by applying 5 ml of water to the water in the Petri dish with spontaneous feeding rasps counted two minutes (baseline water test). Second, 5 ml of the CS was applied to the surrounding solution for 2 minutes and the feeding response was measured (CS test). Finally, 5 ml of the US was applied to the surrounding solution, and the response was measured (US test). By subtracting the baseline water result from the amyl acetate and sucrose solutions result, respectively, a CS-baseline and US-baseline scores were devised and were used for statistical analysis.

6.2.2 Analysis and statistics

Conditioned and unconditioned response scores were calculated by subtracting the baseline rasping rates in water from the rasping rates after the application of the CS and US, respectively. Multiple comparisons were made using ANOVA. Where post-hoc analysis was used, Bonferroni’s correction was used for each assumption.
6.3 Results

As ethanol has not been used in behavioural research in *Lymnaea stagnalis* before, prior to any insightful experimentation, extensive pilot experiments were designed and performed to establish what the most suitable application dose and time parameters would be for these studies. The first section of this chapter presents the results of these pilot studies. These are then followed by the presentation of the findings concerning the effects of ethanol on different aspects of learning and memory, including the state dependence of memory retrieval.

6.3.1 Establishing the optimal dose and time for use in the behavioural experiments

6.3.1.1 Specific Methods

The investigation into a suitable dose was designed to investigate the effects of various doses of ethanol on the snails basic behaviour (initial reaction to the injection, locomotion and feeding). Prior to these experiments a number of additional pilot studies were undertaken to establish a somewhat ‘comparable’ level of ethanol in the snail as in mammalian studies. However, due to the snail having a disproportionate weighted shell relative to its body, calculating the weight of the snails’ body is impossible without having to remove the shell first – which would not be reversible. To lessen the effect that this might have on end concentrations of ethanol, the experiments used as close a size and age of snails as possible, this goes some way to mitigating any difference in end ethanol concentration. A further complication is that even if in vivo concentrations were to be compared they would not be so similar. Since in the mouse the alcohol is largely limited to the blood, whereas in the snail it is freely diluted into the lumen. If the ethanol concentration were to be represented in mM rather than g/kg in the mouse, then calculating the injection volume per weight of the animal (to keep the concentration relevant) would still show the same error. Moreover, these set of experiments looked to other invertebrate models who have also used ethanol in behavioural studies, these also use mM rather than g/kg, whereas the mammalian researchers have adopted the g/kg expression (Sabeti, 2011, Sabeti and Gruol, 2008, Sanchez-Roige et al., 2014, Berry and Matthews, 2004, Silvers et al., 2003, Lee et al., 2009, Pohl et al., 2012, Scholz and Mustard, 2013, Mustard et al., 2008).
No snails were conditioned for this experiment, instead they were injected with a group relevant concentration of ethanol and then allowed to acclimatise to the water in a Petri dish, alone, for 15 minutes. Afterwards, testing began with 5 ml of water being added to the Petri dish and their response was recorded for 2 minutes (baseline test). Immediately after the baseline test, 5 ml of a sucrose solution was applied and the response was recorded for a further 2 minutes (US test). This experiment consisted of 6 groups, who were injected with the following ethanol concentrations; 1000mM ethanol (test day 1 \((N = 13)\), test day 2 \((N = 11)\), 500mM ethanol \((N = 14)\), 250mM ethanol \((N = 15)\), 100mM ethanol \((N = 14)\), saline \((N = 15)\) and un-injected controls \((N = 15)\).

Concurrently to investigating dose, an investigation into the most suitable time-frame was also being performed on different snails. In this experiment the snails were tested after an injection of 250mM ethanol using one of the following 5 post-injection time windows; 15, 30, 45, 60 and 75 minutes. Each animal was assigned to a group once they were collected and food deprived for two days prior to testing. Upon testing, each animal received a 250mM injection of ethanol and was placed alone into a Petri dish for the designated duration. Once the specific post-injection time point was reached, a water test and a US test were performed respectively. This experiment had the following group sizes; 15 minutes \((N = 12)\), 30 minutes \((N = 14)\), 45 minutes \((N = 15)\), 60 minutes \((N = 15)\), 75 minutes \((N = 15)\).

6.3.1.2 Results

On Test day 1 the investigation of dose was performed without prior ethanol/saline injections. The US response on Test day 1 established a baseline for the effect day, Test day 2. The US response rates from test day 1 displayed equal responses between the groups \((p > 0.05)\), demonstrating no bias in rasping ability (Fig 6.1). Test day 2 of the investigating dose experiment revealed that only 1000mM ethanol injection significantly inhibited the response to the US (grouped ANOVA \(p < 0.05\); individual t-tests: 1000mM v control, \(p < 0.05\)). All of the other ethanol concentrations did not produce an effect when compared to 1000mM \((500mM, p < 0.05; 250mM, p < 0.05; 100mM, p < 0.05)\)

The results from investigating a suitable time frame showed comparable responses to the US in all time windows \((p > 0.5)\) (Fig 6.1). The time-frame of 30 minutes pre-training
injection was used as previous work with *Lymnaea* has used the same time window for injections of drugs (Wan et al., 2010, Kemenes et al., 2006b, Kemenes et al., 2006a).

### 6.3.1.3 Interim Conclusions

From these results it was possible to conclude that 1000mM ethanol is too high a concentration for behavioural investigation (there was increased mortality in the 1000mM ethanol group, likely as a result of the injection). Likewise, although the snails treated with 500mM ethanol performed the task well, they were visibly affected by the injection, many of the snails would remain in their shells for the entire post-injection resting duration (a sign of distress). Therefore, 250mM was chosen as the concentration to be used to investigate the effects of ethanol in *Lymnaea*. Moreover, there was evidence of comparable ethanol concentrations in other invertebrate models of behavioural learning and memory experiments (Mitchell et al., 2007, Davies et al., 2003a), and also relevant concentrations in *Lymnaea* electrophysiology (Silver and Treistman, 1982, Treistman and Wilson, 1987).
Figure. 6.1. Establishing the optimal dose and time for ethanol experimentation. (Left) The response to a 2 minute application of the unconditioned stimulus (US) after injection of various ethanol concentrations (1000mM - 100mM). The results of test day 1 showed no bias to the US between the groups ($F_4, 70 = 1.178, p > 0.05$). An ANOVA revealed a significance between groups ($F_4, 70 = 17.967, p < 0.05$). 1000mM ethanol inhibited responding to the US ($T_{26} = 7.715, p < 0.05$). No other concentration showed any difference in the US response (500mM: $T_{24} = 6.350, p < 0.05$; 250mM: $T_{26} = 7.961, p < 0.05$; 100mM: $T_{25} = 7.057, p < 0.05$). (Right) A graph displaying the response to the US after an injection of 250mM ethanol with varying duration denoted by group (15 - 75 minutes). There are no differences between US response rates at different post-injection testing times. Error bars indicate ±SEM.
6.3.2 The effect of ethanol on memory consolidation

One way which ethanol could be affecting memory in general is by interfering with consolidation, the process that transitions STM into LTM. In *Lymnaea* the consolidation pathway has been extensively studied and so it provides a good model to study the effects of ethanol on memory.

6.3.2.1 Specific Methods

Training was performed using the standard classical conditioning protocol with the addition of an injection 30 minutes after training (Fig 6.2). This experiment had the following group sizes; ethanol-injected trained (N = 15), un-trained (N = 15) and control (N = 15).

6.3.2.2 Results

When ethanol was injected 30 minutes after training, there was a significant effect on later retrieval among the groups (p < 0.05) (Fig. 6.3). However, post-hoc t-tests indicated that this difference was between the Control and Un-trained groups (p < 0.05), and reflects a trained vs un-trained group difference rather than an inhibitory effect resulting from ethanol injection. A sucrose test was performed immediately after the CS test showing equal responses between all groups. This finding showed that post-training ethanol treatment did not affect the ability of the snails rasping response to the US and thus further confirmed that the effect between groups was due to training.

6.3.2.3 Interim Conclusions

To conclude, there was insufficient evidence to suggest that ethanol reduced the response to the CS indicating impaired memory consolidation.
Figure 6.2. A diagram displaying the training and testing protocols for the investigation of the effect of ethanol on memory consolidation. Training: All groups experienced a 15 minute acclimatisation period. The ethanol and saline groups are then presented with the conditioned stimulus (CS), 30s later the unconditioned stimulus (US) is presented for the remaining 2 minutes. At this point the snail is removed and waits 15 minutes before the injection of ethanol. The untrained group is only presented with the US for 2 minutes. Testing: All groups are tested in the same manner. They are allowed 15 minutes to acclimatise, then a baseline recording is taken during the 2 minutes proceeding water presentation. This is followed by a 2 minute period following CS application, and a further 2 minutes following US application. During all testing instances the consumption is measured by viewing the amount of rasps (eating behaviour).
Figure 6.3. The effects of ethanol on memory consolidation. Both groups received injections 30 mins after pairing the conditioned stimulus (CS) and unconditioned stimulus (US) to investigate the effects of ethanol on memory consolidation. A One-way ANOVA revealed a difference between all groups ($F_2, 44 = 6.237, p < 0.05$). However, this significance was found to be between the saline and untrained groups ($T_{28} = 3.287, p < 0.05$), indicating a difference between the effect of training versus non-training. The score of the ethanol group is insignificantly lower than that of the trained saline controls and insignificantly higher than the untrained group. Clear bars display the score towards the US, all groups do not differ significantly. All error bars display ±SEM.
6.3.3 The effect of ethanol on memory retrieval

Another possible way in which ethanol could be affecting memory is by effecting retrieval. The following set of experiments used a well-established protocol for the testing of retrieval.

6.3.3.1 Specific Methods

Training was performed using the standard classical conditioning protocol. Testing was performed twice, over two days and included injections in the saline and ethanol groups 30 minutes prior to testing (Fig 6.4). The additional test day was included to act as a confirmation of the test day 1’s result. This experiment had the following group sizes; Ethanol (test day 1 (N = 38), test day 2 (N = 31), Saline (test day 1 (N = 35), test day 2 (N = 31) and an Un-trained group (test day 1 (N = 33), test day 2 (N = 26).

6.3.4.2 Results

6.3.3.1 Test Day 1

Test day 1 revealed an interesting result, both the Ethanol group and the Un-trained group responded less than the Saline group (p < 0.05) (Saline v Ethanol, p < 0.05; Saline control v Un-trained, p < 0.05) (Fig 6.5). This indicated that 250mM ethanol injection, 30 minutes before testing, inhibits retrieval of a previously learnt associative memory. Moreover, there was no effect of ethanol in the US test (p > 0.05), shown by all groups achieving high and comparable results. Also, the CS score shown by the ethanol group was well matched to that of the un-trained group, further indicating that ethanol had inhibited retrieval.

6.3.4.2 Test Day 2

Test day 2’s experiments were performed 24 hours after Test day 1. In contrast to test day 1’s result, the ethanol group displayed a higher response than the un-trained group (p < 0.05) (Ethanol v Un-trained, p < 0.05) (Fig 6.5). When the individual groups were compared between the two test days it was seen that both the ethanol and the un-trained
group increased their response on the second day compared to the first day (Ethanol group day 1 v day 2, p < 0.05; un-trained group day 1 v day 2, p < 0.05).
Ethanol & Saline  Untrained

Training

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Test Day 1

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Test Day 2

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Figure 6.4. The protocol for the effects of ethanol on memory retrieval experiment. Training: Both the ethanol and saline treated animals were trained to associate the conditioned stimulus (CS) with the unconditioned stimulus (US), whereas the untrained animals were not. The same test was performed on consecutive days and consisted of the appropriate injection and the animal being placed into the experimental petri dish for 30 minutes. Testing began with 5ml of water being applied to the surrounding solution, then 2 minutes later the CS, then 2 minutes after that the US was applied. Raspig rate is recorded for each 2 minute period.
Figure 6.5. The effects of ethanol on memory retrieval. To test for the effects of ethanol on retrieval, ethanol was injected 30 minutes before retrieval, 24 hours after training. (Left) Test day one. The saline group displayed a significantly greater response to the conditioned stimulus (CS) than the ethanol ($F_{2, 203} = 7.981$, $p < 0.05$) ($T_{71} = 3.178$, $p < 0.05$) and untrained groups ($T_{58} = 3.340$, $p < 0.05$). The ethanol group responded highly similarly to the untrained group. The response rates to the unconditioned stimulus (US) are similar between all groups, suggesting no group was impaired in its ability to rasp. There was no effect of injection on response to the US ($F_{2, 105} = 2.524$, $p > 0.05$). (Right) Test day two. On the second test day testing was performed 24 hours after test day 1. In contrast to day 1’s result, the ethanol group displayed a higher response than the un-trained group ($F_{2, 87} = 4.374$, $p < 0.05$); Ethanol v Un-trained, $T_{55} = 2.918$, $p < 0.05$). The ethanol group show a much greater response to the CS when compared to test day 1 ($F_{1, 68} = 33.801$, $p < 0.05$), as did the untrained group ($F_{1, 58} = 4.126$, $p < 0.05$). The saline group remained consistent between test days. Error bars indicate ±SEM.
6.3.3.3 Interim Conclusions

These experiments demonstrated a difference in the level of responses to the CS on the two test days. But what caused the increase in both the untrained control group and the ethanol-treated trained group between the two days? In the case of the un-trained group, having the US test immediately after the CS appears to have conditioned the CS. Even though the addition of the US is 2 minutes after the CS, the CS is still present immediately before, and during the application of the US.

As for the ethanol-treated trained group, it could be hypothesized that the observed increase in the conditioned response on test day 2 is due to context-affected learning during Test day 1. The first conditioning trial in this group took place in the absence of ethanol (Fig. 6.4) and the animals in this group failed to retrieve the memory when it was tested 30 minutes after ethanol treatment. During Test day 1 the re-pairing of the CS and US (although delayed) has been shown to induce learning in the un-trained group and therefore it should also do so in this group, however this time the animal would have learnt after an ethanol injection. When the snails are tested 24 hours later, after an ethanol injection in Test day 2, they are able to retrieve because their “state” is the same.
6.3.4 The effect of ethanol on state-dependent associative memory

As a follow-up to the retrieval experiment a further experiment without the two testing days was conducted to elucidate whether ethanol was simply interfering with acquisition or retrieval of the memory formed after an ethanol injection was state-dependent. Additionally, this protocol also allowed for the investigation into the effects of ethanol on acquisition.

6.3.4.1 Specific Methods

Training was performed using the standard classical conditioning protocol (Section 2.2 and 6.2), with the addition of the injections 30 minutes prior to training. Testing was performed in the same manner as the retrieval experiment, but testing only occurred on a single day.

This experiment had the following groups; Acquisition-Retrieval (N = 15), this group received an ethanol injection 30 minutes before training, and again before retrieval; Acquisition (N = 13), this group received an ethanol injection 30 minutes before training, and a saline injection 30 minutes before testing; Retrieval (N = 13), this group received a saline injection 30 minutes before training and an ethanol injection 30 minutes before testing; Control (N = 13), this group received a saline injection 30 minutes before both training and testing (Fig 6.6).

6.3.4.2 Results

An ANOVA revealed a clear effect of treatment (p < 0.05) (Fig 6.7) and that this was due to ethanol inhibiting retrieval in the both Retrieval (Control v Retrieval, p < 0.05; Acquisition-Retrieval v Retrieval, p < 0.05), and Acquisition (Acquisition-Retrieval v Acquisition, p < 0.05) groups. However, there was no inhibition in the Acquisition-Retrieval group (p > 0.05). Moreover the comparable scores between the Acquisition-Retrieval and Control groups suggest that the Acquisition-Retrieval group is performing equally as well as the controls even after receiving two ethanol injections.

To confirm that this was not a form of single-trial learnt motor-tolerance to ethanol, and that both the acquisition and the retrieval group were impaired in their response to the CS alone, a US test was incorporated after the CS test. All groups responded well to the
US and showed a high response level (p > 0.05). Therefore it can be concluded that the reduced responses observed in the ‘Acquisition’ and ‘Retrieval’ groups were not due to the effect of ethanol on motor ability, and that it is due to an impairment of memory by ethanol.

6.3.4.3 Interim Conclusions

From these results it is possible to conclude that ethanol administered before retrieval impairs memory recall. Another conclusion is that ethanol administered before training also impairs memory recall, so long as ethanol is not present at the time of retrieval. However, when ethanol is administered both before training and at the time of retrieval, recall matches the saline injected controls. This confirms the previous hypothesis that ethanol can create state-dependent memories in *Lymnaea*. This is the first experimental evidence for ethanol mediated state-dependent associative memory in any invertebrate model.

This experiment was designed to directly test for a possible ethanol state-dependent effect of retrieval in *Lymnaea*. However, this experiment also provided further support for the previous finding that ethanol inhibits retrieval if 250mM is injected 30 minutes prior to memory recall. Also, it showed that if a snail is trained 30 minutes after an ethanol injection, it is inhibited in retrieval if it is injected with saline 30 minutes before retrieval. Interestingly, this was found not to be an inhibition of acquisition since if the snail was trained and tested after an ethanol injection it was able to retrieve, and displayed a response matching that of the saline injected controls.
### Figure 6.6. Protocol for the investigation of the effects of ethanol on state-dependent associative memory.

Each group name denotes its injection procedure. The first word indicates the injection received before training. The second word indicates the injection received before testing. Training consists of the snail being placed into a petri dish immediately after the appropriate injection. 30 minutes later the 5ml of the conditioned stimulus (CS) is applied, after 2 minutes 5ml of the unconditioned stimulus (US) is also presented to complete the conditioning. Testing occurs 24 hours later, and consists of being placed in a petri dish with 90ml of water for 30 minutes before water is applied, two minutes later the CS is applied, after two further minutes the US is applied.
Figure 6.7. The effect of ethanol on state-dependency associative memory. There is a clear difference between the groups’ response to the conditioned stimulus (CS) ($F_{3, 53} = 8.222, p < 0.05$), with both acquisition (Acquisition-Retrieval v Acquisition, $T_{24} = 3.051, p < 0.05$) and retrieval groups displaying lower responses to the CS (Control v Retrieval, $T_{26} = 4.058, p < 0.05$; Acquisition-Retrieval v Retrieval, $T_{24} = 4.718, p < 0.05$). The acquisition-retrieval group displayed a response to the CS comparable to that of the saline group, showing higher values than the other ethanol receiving groups. All groups’ response to the US were comparable. Importantly, the responses to the unconditioned stimulus (US) in the groups that responded poorly to the CS were high, suggesting ethanol was not influencing motor actions. Error bars indicate ±SEM.
6.3.5 Pharmacological investigation of ethanol state dependency

Based on the results of the previous two experiments, it was decided that pharmacological investigation into the mechanisms of ethanol state-dependency would provide greater insight into how ethanol affects memory. It was hypothesised that by utilising various drugs that, in some way, mimic the effects of ethanol (either sharing a similar molecular action, or inducing a similar emotional state) could also retrieve a memory that was conditioned after an injection of ethanol.

6.3.5.1 Specific Methods

The experiment designed to test this hypothesis followed the same protocol as the acquisition and state-dependency experiment. However, this experiment substituted the ethanol or saline injection before testing with treatment with a variety of pharmacological agents (NMDA, Ketamine, GABA and Picrotoxin). All drug concentrations were first tested for suitability of dosing in a similar methodology to the experiment Investigating dose.

6.3.5.2 Results: Involvement of NMDAR in ethanol state-dependent retrieval

NMDA receptors are a well-established target for ethanol. Already this thesis has shown that ethanol can modulate LTP in mice, likely by interactions with NMDA receptors. By investigating both an agonist and antagonist of the NMDA receptor it was possible to investigate the involvement of the NMDA receptor in ethanol state-dependency in Lymnaea.

NMDA works as a potent NMDA receptor agonist and has been shown to be effective in Lymnaea, including being involved with motor neurons of the feeding system (Ha et al., 2006). Ketamine is a widely used non-competitive NMDA receptor antagonist, and doses around $10^{-7}$ to $10^{-5}$ M have been shown to have both stimulatory and sedative properties in Lymnaea (Woodall and McCrohan, 2000), as well as being able to block LTM formation in Lymnaea (Browning and Lukowiak, 2008). In chapter 4 the NMDA receptor antagonist MK-801 was used to inhibit the induction of LTP. At the time of this experiment it was calculated that MK-801 – at the concentration needed – would have had to utilising a vehicle such as DMSO (Dimethyl Sulfoxide) to become solute and prepared for injection (Wan et al., 2010). This would have created significantly more groups to run as
controls and therefore unpractical. Additionally, although MK-801 is a potent NMDA receptor antagonist it does have some side-effects when used in behavioural studies, and is generally used in the induction of psychosis (Andine et al., 1999). For this reason and others MK-801 is typically avoided in clinical experiments/environments (Olney et al., 1989). Moreover, it was considered that ketamine may better emulate the intoxication effects of ethanol and could possibly allow for an investigation of internal-context retrieval mechanisms.

NMDA

Experimentation with injection of NMDA revealed a source of significance in the CS response levels among the four experimental groups (p < 0.05) (Fig 6.8). However, the post-hoc analysis revealed only a statistical trend between the SN and Saline group (p > 0.05). Although seemingly weaker, the retrieval in the ethanol – NMDA group was not significantly impaired when compared to the ethanol – ethanol group (p > 0.05). Therefore NMDARs cannot be confirmed either way of their involvement in ethanol state-dependent memory.

Ketamine

The investigation of the NMDA receptor antagonist ketamine revealed that injection before retrieval results in a significantly reduced CS response in saline trained snails and a trend for a reduction in CS response in the ethanol trained snails (p < 0.05; saline – saline v saline – ketamine, p < 0.05; ethanol – ethanol v ethanol – ketamine, p > 0.05) (Fig 6.8). These findings suggest that blocking NMDA receptors has an inhibitory effect on retrieval. However, it cannot be ruled out that ketamine plays some role in ethanol state dependent memory.
Figure 6.8. Pharmacological investigation of state-dependency: NMDA (Left) and Ketamine (Right). (Left) Both Saline-NMDA (SN) and Ethanol-NMDA (EN) groups did not match the responses of either the saline or ethanol groups ($F_{3, 77} = 3.127, p < 0.05$). However, when post-hoc independent t-tests were performed, a statistical trend could be revealed between the SN and Saline group ($T_{37} = 2.625, p = 0.078$). Although the EN group appeared to be show lower levels than the Ethanol group it was insignificant ($T_{37} = 1.649, p > 0.05$). (Right) Neither Saline-Ketamine (SK) nor Ethanol-Ketamine EK groups matched the levels of either the Saline or Ethanol groups ($F_{3, 76} = 4.505, p < 0.05$). Ketamine injected into saline injected trained snails produced a significant inhibition of retrieval ($T_{37} = 2.937, p < 0.05$). When injected into ethanol injected trained snails it produced a close to significant trend ($t_{37} = 2.715, p = 0.06$). Error bars indicate ±SEM.
6.3.5.3 Results: The role of GABA receptors in ethanol state-dependent retrieval

Ethanol has a known inhibitory interaction with GABA receptors and is known to be implicated in many of ethanol’s effects (Chandler, 2003, Fleming et al., 2013, Yang and Ma, 2011, Hunt, 1983, Tsang et al., 2007). This provides a reason to suspect a role for GABA receptors in ethanol state-dependent retrieval.

The amino acid neurotransmitter GABA is a potent non-selective GABA receptor agonist, and has already been used in *Lymnaea* at relevant concentrations (Moccia et al., 2009). PTX is a non-competitive GABA antagonist and has also been used in the *Lymnaea* system before (Moccia et al., 2009). By using both GABA modalities the aim was to investigate the role of the GABA receptor in ethanol state-dependent retrieval.

**GABA**

The investigation using GABA revealed no statistical difference among the four experimental groups (p > 0.05) (**Fig 6.9**). Although there was a tendency for the CS-induced responses to be weaker after GABA treatment it was insufficient to conclude either that GABA blocked retrieval or that it did not play a role in ethanol state-dependent memory.

**Picrotoxin**

Similar to GABA, the investigations with PTX revealed no statistical difference in the CS score among the four experimental groups (p > 0.05) (**Fig 6.9**). Although there was a tendency for the CS-induced responses to be weaker after PTX treatment the results were unable to conclude either that PTX blocked retrieval or that it did not play a role in ethanol state-dependent memory.
Figure 6.9. Pharmacological investigation of state-dependency: GABA (Left) and Picrotoxin (Right). (Left) In both the Saline-GABA (SG) and Ethanol-GABA (EG) groups responded less than the Saline and Ethanol groups ($F_3, 76 = 1.172, p > 0.05$). This suggests that GABA is not able to retrieve the ethanol induced state-dependence. (Right) Both the SP and EP groups also did not replicate the responses seen in both the Saline and Ethanol groups ($F_3, 75 = 2.390, p = 0.076$). Error bars indicate ±SEM.
### 6.3.5.4 Interim Conclusions

None of the drugs used in this set of experiments was able to facilitate the retrieval of the ethanol state-dependent memory. The drugs themselves did not appear to greatly impair the performance of the snails in any form, and generally the performance between the drug-injected saline and ethanol-trained snails was well matched. This may suggest that the pathway behind ethanol state-dependent memory is more complex than a single receptor interaction.
6.4 Discussion

6.4.1 Establishing a suitable ethanol model for use in Lymnaea stagnalis

Since these were the first experiments testing the behavioural effects of ethanol on Lymnaea stagnalis, it was first necessary to establish a suitable ethanol concentration to inject, and post-injection duration to use before testing/training commenced. This investigation aimed to find an ethanol concentration that was high enough to act as a model for the high BEC seen in human binge drinkers and AIE treated rodents that suffer from memory dysfunction, but low enough not to cause any unwanted extreme side-effects. Although sometimes difficult, and often unwarranted to generalise concentrations between different animal models, consistency between the ethanol concentrations used here in Lymnaea and the concentrations used in other invertebrate species would be a major advantage.

Immediately after high concentration ethanol injections such as 1000mM and 500mM, there were some unusual behaviours noticed in the snails such as withdrawal into their shells, penile erections and unusual bodily extensions. After multiple pilot experiments the ethanol concentration chosen was 250mM. It was felt that this concentration was high enough to alter behaviour, but did not result in any apparent immediate discomfort or gross inability of functioning.

It was also necessary to establish a suitable time-window. An ideal a time would be where ethanol was having its pharmacological affect without it causing any unwanted extreme stimulatory or sedentary effects. The snail’s ability to respond to the experimental US was tested at various time durations after a 250mM ethanol injection to rule out possible time-dependent stimulatory or sedative behaviours. There was no significant effect of time on US response after ethanol injection. It was concluded that a 30 minute pre-training injection was appropriate for the planned further experiments. Moreover, much of my previous work with Lymnaea had used the 30 minute pre-training or pre-testing time-window for injection protocols, which bestows confidence of its suitability.
6.4.2 The effect of ethanol on memory consolidation

Given that there is little to no current published research on the effects of ethanol on memory consolidation in invertebrates, comparison has to be made in vertebrates. In some ways this experiment is similar to the mouse operant study in the previous chapter, where it was hypothesised that ethanol could be interfering with the consolidation of the operant lever pressing task. There have been several reports in mammals that post-training injections of ethanol can inhibit consolidation (Aversano et al., 2002, Castellano and Populin, 1990, Castellano and Pavone, 1988, Ryabinin et al., 2002) (these are discussed in detail in the previous chapter). It was initially hypothesized that ethanol would inhibit retrieval (it inhibited both retrieval and acquisition) however in this experiment the results were inconclusive. The ethanol group response to CS was not significantly weaker than in the saline-trained group nor significantly higher than in the non-injected untrained group.

This result could be interpreted in a number of ways. One possibility is that ethanol does affect consolidation, but was not expressed in this experiment. Another is that the expression was not fully inhibitory and this is why the CS response levels did not match the untrained or significantly different from the trained snails. Otherwise, ethanol does not affect consolidation in this experiment. If the latter is true then this could provide great insight into by which mechanism ethanol is having its effect on in the case of retrieval and state-dependency.

6.4.3 The inhibitory role of ethanol on acquisition and retrieval of associative conditioning and ethanol state-dependence

From both the retrieval and state-dependency experiments it is clear that ethanol is affecting memory and inhibiting retrieval, unless ethanol is also present during acquisition or secondary acquisition / reconsolidation whereby the memory will again become retrievable. Day 2 of the retrieval experiment complicated the conditioning process. The testing of the US two minutes after the CS in day 1 (both CS and US are fluids, the CS remains in the solution as the US is presented) could be seen as a form of delayed conditioning (Sweatt, 2010). However this additional pairing of the CS and US may have a weaker contingency and could be considered an unreliable duration between the two stimuli for successful conditioning to occur. This secondary delayed conditioning could have a contingency anywhere between zero seconds two minutes. Interestingly,
the ethanol group showed much higher CS response in day 2 than in day 1. The further training was also shown to have increased the CS score response in the un-trained group in test day 2 – in which case it could be argued that they are not ‘un-trained’. However the additional training did not further increase the response in the control group. The un-trained group could now be considered to have been trained during day 1’s testing. Likewise, the saline group could have had its original training modified, a high contingency pairing could increase the conditioned behaviour while a low contingency could lead the behaviour towards extinction (Sweatt, 2010). However, the memory has not become extinct, given that the control group responded equally well in test day 2 as they did in test day 1. It is likely the case then that the ethanol group received additional conditioning whilst under the effects of ethanol. Then when tested on the subsequent day after receiving an ethanol injection, the snails were able to retrieve the memory. This process of only being able to retrieve a memory if it is in the same context as acquisition is called state-dependent memory.

Evidence has been provided that ethanol inhibits acquisition and retrieval of a memory acquired under saline conditions. It also suggested that – although the experiment was relatively inconclusive – consolidation is unaffected by ethanol (at least at the concentration used – 250mM). It is known from extensive work on the molecular mechanisms of associative memory in *Lymnaea* by the Kemenes and Benjamin laboratories (reviewed in (Kemenes, 2013)) which molecular components are instrumental in the different phases of the memory process in *Lymnaea*. Many of the molecular components that are utilised in acquisition are also necessary in consolidation, such as PKA, MAPK and CaMKII (Kemenes, 2013, Spruston, 2007). If ethanol is having its effect through a target molecule then it would be expected to be targeting a component that is involved in acquisition but not consolidation. In *Lymnaea* this would suggest that ethanol is effecting either PACAP (Pituitary adenylate cyclase-activating polypeptide) or NMDA receptors, since these are the two identified molecules involved with acquisition but not involved with consolidation. Ethanol’s involvement with NMDA receptors has received significant attention, ethanol is known to have varied interactions with NMDA receptors (Hendricson et al., 2004, Hoffman et al., 1990, Mulholland et al., 2009, Chu et al., 1995, Xu et al., 2008, Allgaier, 2002, Schummers and Browning, 2001), and is also known to inhibit NMDA-dependent hippocampal LTP (Givens and McMahon, 1995, Schummers et al., 1997, Nelson et al., 2005, Fujii et al., 2008, Izumi et al., 2008, Mishra et al., 2012).
Interestingly ethanol’s inhibitory effect on retrieval has been also noted in honey bees, where ethanol was found to inhibit retrieval by modulating acquisition. Moreover the same bee study found that when ethanol was consumed after conditioning that there was no effect on subsequent retrieval, like the ethanol group in the consolidation experiment (Mustard et al., 2008). The author offers speculation that NMDA receptors may be ethanol’s target in the inhibition of acquisition. Although it should be stated that their arrival at that conclusion was based on many reasonably unrelated studies, such as ethanol’s action on NMDA receptors in the hippocampi of rodents, and that recently, NMDA receptors had been found in the honey bee (Zannat et al., 2006). However, ethanol also only affected acquisition and not consolidation in the honey bee, using in a similar experimental design, albeit in a different species (Mustard et al., 2008). This does add support to the involvement of NMDA receptors in the action of ethanol on memory.

The most interesting finding in this series of experiments was that of ethanol-induced state-dependency. This was the first documented occurrence of ethanol state-dependent associative learning in invertebrates to the knowledge of the author at the time the experiment was performed. There is however some evidence for ethanol state dependence in a sensory adaptation response in *C. elegans*, which usually adapts, or shows a reduced behavioural response to an olfactory stimulus after pre-exposure to the specific stimulus. A study showed that if adaption to an olfactory stimulus was acquired after ethanol administration, then the adaption is only retrievable once ethanol is administered again (Bettinger and McIntire, 2004).

The notion of ethanol state-dependence, learning whilst intoxicated and then only remembering when again intoxicated, has existed colloquially for some time. However, understanding whether that was through colloquial hear-say, or from scientific process is difficult. Possibly the earliest rigorous study of ethanol state-dependence showed that human subjects performed better in multiple memory tasks if the subject was intoxicated in both learning and testing sessions than in either one alone (Goodwin et al., 1969, Oei and Young, 1986). However, performance in recollection tasks of state-dependent memories could be improved by prompting the individual or providing cues (Petersen, 1977). Others have found ethanol state-dependent effects in humans on explicit but not implicit memory tasks (Duka et al., 2001).

More empirical data comes from later experimentation on rodents, where i.p injections of ethanol (0.25, 0.5 and 1g/kg) impaired memory retrieval in a dose-dependent manner.
However, when a pre-test administration of 0.5 or 1 g/kg ethanol was used in the 0.5 or 1 g/kg ethanol trained group, state-dependent retrieval was observed (Rezayof et al., 2007, Rezayof et al., 2008a, Rezayof et al., 2010b, Zarrindast et al., 2013). Ethanol state-dependency does appear to have dose-dependent upper boundaries. One study found ethanol state-dependency at 1.2 g/kg but not at 2.4 g/kg. Furthermore, the 2.4 g/kg dose continued to have amnesic effects in all conditions (Sanday et al., 2013). Interestingly, the contextual state of a consolidated memory can be altered during the labile phase of reconsolidation (Nader and Einarsson, 2010, Sierra et al., 2013) suggesting that while a memory is being reformed it is sensitive to additional contextual states (Sierra et al., 2013). It is also possible to convert a previous memory or conditioning into a state-dependent one (Flint et al., 2013, Sierra et al., 2013). This could explain the initial state-dependent effect seen in the first retrieval experiment (6.3.3). Where snails were trained under saline and tested after ethanol, the ethanol may well have had persisted during the consolidation phase such that the memory trace is being reconsolidated in the context of ethanol. This could have been tested for by also performing two test days on the consolidation experiment. If the snails were trained under saline conditions then the memory trace reconsolidated under ethanol conditions, then underwent subsequent testing under saline conditions, they could be expected to display an inability to retrieve.

The Rezayof laboratory is involved with most of the recent studies into ethanol state-dependent learning in mammals have spent a number of years studying the underlying cellular and molecular mechanisms. Confusingly, they propose and show multiple pieces of evidence for various different neurotransmitter or molecular mechanisms that they believe to be involved in ethanol state-dependence with every new publication. With little to no mention of the previous discussions other than ethanol’s proven ability to impair learning and form memories in a state-dependent fashion. For example they have described the effects of ethanol state-dependent memory – typically in the dorsal hippocampus of mice – to have some involvement with, or be able to be recovered by the following; dopamine (Rezayof et al., 2007, Piri et al., 2013), acetylcholine (Rezayof et al., 2008a), NMDA receptors (Rezayof et al., 2008b), nicotine (Rezayof et al., 2010a, Alijanpour and Rezayof, 2013, Alijanpour et al., 2015), Beta-adrenoreceptor (Zarrindast et al., 2013), and CREB (Alijanpour et al., 2015). They also report that L-arginine co-administration with ethanol inhibited state-dependency (Rezayof et al., 2010b). These findings may reflect the complexity of ethanol as a drug and its interactions with many receptors and molecular functioning. Or, perhaps some part of their experimental
procedure is causing ethanol state-dependency. This makes any confident conclusion derived from this series of experiments difficult.

In an attempt to better understand and characterise this new form of memory in *Lymnaea*, drugs were used that are both similar in behavioural effect and molecular action to ethanol to try and successfully facilitate the retrieval of a memory acquired under the influence of memory. However, no single drug was able to fully replicate the effect of ethanol and allow the retrieval of the ethanol state-dependent memory. These experiments were performed before the publication of studies indicating that nicotine, dopamine or acetylcholine could promote the retrieval of ethanol encoded state-dependency (Alijanpour and Rezayof, 2013, Alijanpour et al., 2015, Rezayof et al., 2008a, Zarrindast et al., 2013), and also these drugs do not tend to cause similar behavioural effects. The aim was to substitute ethanol at the time of retrieval for another drug that could mimic either the pharmacological or the psychological effects of ethanol, in the hope to reveal a shared molecular mechanism. By this hypothesis, the drug should not only retrieve the memory of ethanol trained snails but it should also inhibit the retrieval of the saline trained snails. If the drug was a perfect substitution, then it would be expected to increase the response in the ethanol trained animals to the level of the ethanol – ethanol group, however a perfect substitution would be unlikely and was not noticed in this case.

Of all of the ethanol – drug groups used in this experiment the highest CS score found was in the ethanol - NMDA group. Even though it was the highest, it was not comparable to the ethanol – ethanol group, suggesting that NMDA could not retrieve an ethanol encoded memory. Additionally, the saline – NMDA group showed a very low level of response after an injection of NMDA, lower than even the naïve group. Showing some – limited – similarities to ethanol state-dependency. The lowest performing ethanol – drug group was ethanol – ketamine. This expresses essentially the opposite effect of NMDA, and inhibits NMDA receptor functioning. This could be seen as indirect support for a role of the NMDA receptor in ethanol state-dependent memory. However, it must be stressed that this experiment was not found to be statistically significant and should therefore be considered highly speculative. The two GABA receptor drugs, GABA and PTX did not show a significantly reduced CS score when compared to the ethanol – ethanol group, neither did they show an increased CS score compared to that of the untrained naïve group. Therefore the involvement of GABA receptors in the observed ethanol state dependent retrieval could neither be confirmed nor ruled out in the present experiments.
6.4.4 Conclusions

To conclude, administration of ethanol is able to block both the acquisition and retrieval of an associative memory in the snail *Lymnaea stagnalis*. Importantly, the inhibition of retrieval by ethanol administration during acquisition can be circumvented by a pre-retrieval injection of ethanol, thus showing a state-dependent relationship of ethanol on associative memory. Pharmacological investigations into the molecular underpinnings of this process revealed mixed results, but did not rule out either NMDA or GABA receptors. Therefore it is sensible to conclude that the memory process by which ethanol has its effect is likely to be complex and may involve multiple receptors and molecular pathways.

An important conclusion based on the behavioural findings presented in this chapter is that to fully understand how ethanol affects memory it would be useful to continue down the reductionist's path and understand how ethanol affects an important modulatory neuron – the CGC – within the learning and memory neural pathway. The CGC is necessary for correct functioning of the type of learning and memory investigated in this chapter. Therefore in the next chapter it is important to test how ethanol affects the electrical properties of an important single cell this would allow for information on how ethanol might affect memory in larger cellular networks.
Chapter 7 – The effects of ethanol on an identified invertebrate neuron involved in encoding long-term memory

The previous chapters investigated the behavioural and circuit level effects of ethanol in a mouse model of spatial learning and operant conditioning and ethanol-induced changes in memory retrieval in a snail model of classical conditioning. However, in order to understand how behavioural and circuit level changes occur after ethanol exposure, it is important to elucidate how it affects individual neurons known to play key roles in specific learning and memory circuitries. One such neuron is the Cerebral Giant cell (CGC), an important serotonergic modulatory neuron of the feeding system of the pond snail *Lymnaea stagnalis* (McCrohan and Benjamin, 1980a, McCrohan and Benjamin, 1980b, Yeoman et al., 1994b, Yeoman et al., 1994a, Yeoman et al., 1996). Importantly, the CGC has been identified as a key neuron of the *Lymnaea* learning and memory circuitry (Kemenes, 2013, Benjamin et al., 2000, Vavoulis et al., 2010, Staras et al., 2002). It has a large cell body and is easily impaled by microelectrodes, allowing for the direct measurement of how ethanol is affecting its electrophysiological properties. This final results chapter provides an insight into how different *in vitro* protocols of ethanol application to the isolated nervous system of *Lymnaea* can affect the CGC at the most fundamental electrophysiological level.

This chapter starts by further discussing *Lymnaea* as a model organism for single cell and small neuronal network electrophysiology, before introducing the relevant literature on how ethanol affects invertebrate neurons. Two experiments are then detailed describing the effects ethanol application, both continuous and intermittent, have on the CGC’s tonic firing pattern and spike characteristics.
7.1 Introduction

7.1.1 *Lymnaea* as a model organism for investigating the effect of ethanol on a single neuron

Ethanol has a highly complex interaction with receptors, individual cells, neuronal networks and whole neuronal systems. To better understand how ethanol affects the whole brain or specific networks of neurons, it is important to understand how ethanol acts on single neurons. The snail *Lymnaea stagnalis* is a well-established model for the study of learning and memory (Kemenes, 2013, Wan et al., 2010, Benjamin et al., 2000). Its relatively simple feeding behaviour can be conditioned and used for the investigation of the cellular and molecular mechanism of memory (Kemenes et al., 1997, Audesirk et al., 1982, Alexander et al., 1984). Along with its other molluscan counterparts, such as *Aplysia* it has been used with great success and has taught us much about the cellular and molecular mechanisms of both non-associative and associative memory (Castellucci et al., 1978, Ha et al., 2006, Kandel, 2012, Kandel, 2009). Other advantages that come with using this model organism is that the neuronal circuitry underling the conditioned feeding behaviour has already been well established. The cells involved with the *Lymnaea* feeding system are well mapped and are easily identified (Kemenes, 2013). After classical conditioning, the neurons involved become modified and show different electrophysiological responses, thus allowing for an interlinked behavioural and electrophysiological approach to be used (Benjamin et al., 2000).

One of the most readily identifiable cell types in *Lymnaea* are the pair of large modulatory serotonergic neurons known as the cerebral giant cells (CGC’s). These cells have a large cell body (~100 µm) which allows for easy insertion of electrodes, and their electrophysiological properties have been extensively studied (see (Staras et al., 2002) for a full characterisation). Moreover, their homologs exist in other molluscan models, and have also been extensively studied, and are known to be highly comparable (Kupfermann and Weiss, 1982, Kupfermann et al., 1979, Morgan et al., 2000). The CGC contains seven different voltage-dependent currents; two sodium, three potassium and two calcium currents (Staras et al., 2002). Of the two Na⁺ channels, one conducts a large transient inward Na⁺ current and the other gives rise to a low-threshold small persistent Na⁺ current. The two outward potassium currents are an A-current, conducted by a fast activating and fast inactivating channel which is inactivated at potentials more positive.
than -40mV and a delayed rectifier potassium current. The latter is activated at more depolarised levels and shows slow activation and inactivation kinetics. Finally, there are two types of calcium channels. The first one gives rise to a low voltage-activated T-type current with fast inactivation kinetics and a low activation threshold while the second one conducts a high voltage-activated calcium current (Staras et al., 2002). The transient Na⁺ current and the K⁺ and Ca²⁺ currents together contribute to the generation and shaping of the CGC action potentials (Staras et al., 2002), while the persistent Na⁺ current has a major role to play in the membrane potential (Nikitin et al., 2008).

Importantly, over a period of 16-24 hours after associative conditioning the CGCs undergo changes leading to the emergence of non-synaptic plasticity: the cell body becomes persistently depolarised by between 5 and 10 mV, increasing the synaptic output of the cell by an intracellular calcium-dependent process (Kemenes et al., 2006b, Nikitin et al., 2013). This depolarisation of the CGC’s is what enables the expression of LTM (Kemenes et al., 2006b, Vavoulis et al., 2010). The depolarisation is modulated by cAMP, which can increase the amplitude of the low-threshold persistent sodium current which is an important factor in the cells membrane potential (Nikitin et al., 2006, Nikitin et al., 2008). However the depolarisation has no effect on the firing frequency or spike shape because it is balanced out by changes in the delayed rectifying potassium current (I₉) and a high-voltage-activated calcium current (I₉VA) (Vavoulis et al., 2010).

7.1.2 Ethanol’s effect on individual neurons

To date, relatively little work has been performed on ethanol’s effects on single molluscan neurons and none of them targeted identified neurons with an established role in learning and memory. Although some data is available in *Aplysia californica* on the MCC, a homolog of the *Lymnaea* CGC, the MCCs never have been investigated for a role in learning and memory. By using a voltage clamp technique it was revealed that 200 – 400mM ethanol produced an increase in the decay constant of the early potassium currents of the MCC (Treistman and Wilson, 1987). It has also been noted that higher concentrations of ethanol (400 – 600mM) induced spontaneous burst firing in otherwise silent neurons (Silver and Treistman, 1982). Another study investigating the biophysical effects of various alcohols showed that 660mM ethanol decreased *Lymnaea* A-type fast inactivating potassium current (Alekseev et al., 1997). 800mM ethanol has been shown to accelerate the rate of decay of post-tetanic potentiation after repetitive electrical stimulation (Brown et al., 1988). The majority of these studies were primarily investigating
the biophysical interaction between membranes and alcohols (ethanol and higher chain alcohols). These points aside, the concentrations used in these experiments are extremely high and physiologically irrelevant to any study where the organism is going to survive. Therefore it is necessary to investigate more physiologically relevant ethanol concentrations on single cell physiology.

Studies attempting to better reflect physiological ethanol concentrations have been performed using the terrestrial snail *Helix aspersa*. 50mM ethanol decreased both the probability of spontaneous action potentials and frequency by enhancement of repolarisation and after-hyperpolarisation (Cooper, 2005). Another physiologically relevant ethanol study used the snail *Helix pomatia*. 200mM ethanol interfered with many of the molecular structures that are involved with snail learning and memory, such as G-proteins, cAMP-dependent protein kinases and PKC. Moreover they suggested that Ca\(^{2+}\) channels were the most sensitive targets to the effects of ethanol (Kerschbaum and Hermann, 1997).

### 7.1.3 Aims and Hypotheses

In the limited number of studies that have investigated the effects of ethanol on single neurons none have investigated what would happen if ethanol was removed and possibly re-applied. From the results in Chapter 4: Investigating the effects of ethanol on hippocampal LTP it is known that different protocols of ethanol application lead to different effects. By using the CGC, a well-characterised cell that is critically involved in the *Lymnaea* feeding and memory systems (Kemenes, 2013, Vavoulis et al., 2010), it is possible to investigate how ethanol affects its underlying electrophysiological properties. For instance, is the effect of ethanol reduced as the total concentration/duration is decrease? Or does a periodic application of ethanol allow for recovery during ‘off’ periods? Or perhaps, intermittent ethanol triggers a different response than a constant perfusion of ethanol?

The main aims of this chapter are:

1. To investigate the effects of a physiologically relevant constantly maintained ethanol concentration on the electrophysiological properties of the CGC.
2. To investigate how the spiking activity of the CGC changes if ethanol is applied using an intermittent protocol.
7.2 Methods

7.2.1 Apparatus

Microelectrodes (10-40MΩ tip resistance) were made from borosilicate glass capillaries (Harvard Apparatus, 1.16mm inner diameter, and 2 mm outer diameter) by using a vertical puller (Narishige). Electrodes were filled using a conducting solutions of 4M potassium acetate, and the tips coated with black drawing ink (Rotring to help with the insertion of the electrodes. The electrodes were held in place by micromanipulators and connected to a headstage Neurolog NL102 preamplifier (Digitimer. Ltd). The signal was digitalised using a Micro 1401 mkII (CED), the digital signal was processed using the Spike2 versions 5.14 software (CED). The cells were viewed using a Motic microscope, and were lit using a Cole Palmer 41723 – Illuminator. A perfusion system was used comprising silicon tubing, steel piping and adjustable valves to fine tune the gravity-assisted flow rate. A Gilson Minipuls3 peristaltic pump was used to remove excess fluid from the system.

7.2.2 Snail brain preparation

The central nervous system (CNS) was removed from the snail by first removing the shell using scissors and then cutting the body in half from starting from just below the mantle towards the foot. The buccal mass was then repositioned to reveal the CNS, the nerves were cut and the CNS removed. Once isolated, the CNS was pinned to the bottom of a sylgard filled small Petri dish, and positioned so that the CGC was accessible. A final layer of connective tissue was removed and a small amount of protease applied for one minute, and then washed out several times with saline.

7.2.3 Ethanol application protocols

Two types of ethanol application protocols were used. These are shown in Fig. 7.1 and described in detail in the relevant Results sections.
Figure 7.1. Diagrams of the protocol of ethanol exposure in the constant ethanol (Top) and intermittent ethanol (Bottom) procedures. (Top) Constant ethanol: The first 15 minutes are a baseline recording period using saline alone. In the last 5 minutes of the baseline period saline perfusion was stopped. 15 minutes after the start of the experiment the ethanol perfusion was started; from this point until the end, ethanol was always perfused when the perfusion system was switched on. The black boxes indicate periods of non-perfusion, however during these periods, ethanol would remain in the solution around the cells. (Bottom) Intermittent ethanol: The first 10 minutes of the experiment are performed under saline conditions and act as a baseline. Grey boxes indicate when ethanol is perfused, white boxes indicate saline perfusions.
7.2.4 Electrophysiological recordings

A 10-40MΩ electrode was inserted into the CGC, which allowed the recording of the membrane potential. Once a stable baseline was achieved ethanol (or saline) was applied in one of the procedures shown in Fig. 7.1.

7.2.5 Analysis and Statistics

Spike parameter data was exported out of Spike2 by using a custom-written script which measured five variables: amplitude (mV), frequency (Hz), half-width (ms), peak-to-trough (ms) and repolarisation (ms) (Fig 7.2). Due to offset level drift and other artefactual changes in the membrane potential doing these long-term recording and perfusion experiments, it was not possible to make a meaningful comparison of this parameter among the different treatment groups.

The exported spike parameter data was analysed in two ways. Value data: shows the raw recorded data grouped into 5 minute time bins. Percentage value: the raw data was further grouped into 15 minute time periods (Fig 7.2). All time periods were normalised to baseline levels, and expressed as a percentage.

Repeated measures ANOVA’s with post-hoc independent and paired t-tests were used to assess significance (where necessary Bonferroni corrections were used for each comparison). If sphericity failed, then a Greenhouse-Geisser correction was used.
Figure 7.2. Diagram of what spike characteristics of the CGC were analysed. Amplitude: voltage difference between the resting membrane potential and the peak of the spike. Repolarisation: the time between maximal hyperpolarisation and return to the original resting membrane potential. Half width: the duration between the rising and falling phases of the voltage midpoints between the resting membrane potential and the peak amplitude. Peak-to-trough: time between the peak amplitude and maximal hyperpolarisation.
7.3 Results

7.3.1 Experiment 1: The effects of constant ethanol application on the CGC

7.3.1.1 Specific Methods

A baseline was recorded for 10 minutes, followed by a non-perfusion period of 5 minutes. Then (in the ethanol group) 80mM ethanol was perfused for 10 minutes followed by a five minute non-perfusion period without wash out. Ethanol was perfused for a further 20 minutes followed by 5 minutes without perfusion, this is then repeated once more to make a total recording period of 80 minutes (see Fig 7.1).

7.3.1.2 Results

Spike Amplitude

There was no overall significant difference in CGC spike amplitude between the groups over time. However, there was a close trend seen in the general reduction of amplitude over time (p > 0.05), but each group did not show a significant difference between the rate of decline (Group by time interaction, p > 0.05) (Fig 7.3). Therefore it is not possible to state that a continuous supply of 80mM ethanol significantly alters the CGC’s spike amplitude.
Figure 7.3. The effects of constant ethanol application of the CGC’s amplitude. (Left) Displays the amplitude in mv grouped into 5 minutes periods. A repeated measures ANOVA revealed no significant differences between the Saline and Ethanol groups (F\(_{1.788, 19.663} = 0.739, \ p > 0.05\)), and did not decline in amplitude significantly (F\(_{1.788, 19.663} = 3.458, \ p > 0.05\)). (Right) Shows the change in amplitude as a change in percentage of baseline over time, the time bins are further grouped. The Ethanol group has a minor - statistically insignificant - drop in amplitude of around 8-10% of its original baseline values (F\(_{1.515, 16.668} = 3.623, \ p > 0.05\)). There was also no statistical difference between group (F\(_{1.515, 16.668} = 0.713, \ p > 0.05\)). Error bars indicate ±SEM.
Figure 7.4. Examples of the difference seen in spike images after ethanol treatment. (A) Constant saline during the baseline period. (B) Constant saline towards the end of a recording. (C) Examples of how the CGC’s spikes look at the start of a recording and (D) at the end of the recording. Here ethanol has noticeably reduced the firing frequency.
Frequency

After the baseline period both groups showed a reduction in firing frequency over time continuously until the end of the recording (Value data, p < 0.05; Percentage data, p < 0.05) (Fig 7.5). Interestingly, the Ethanol group showed a greater reduction in frequency than the Saline group (Value data group by time interaction, p < 0.05; Percentage data group by time interaction, p < 0.05). Post-hoc t-tests of the percentage data revealed that frequency decreases significantly in both groups between time bins 1 and 2 (Ethanol group, p < 0.05; Saline group, p < 0.05).

Half-Width

The results from measuring Half-Width show that it reduces with time in both groups (Value data, p < 0.05; Percentage data, p < 0.05). Moreover, it was found that 80mM ethanol reduced the half-width duration more than the saline solution alone (Percentage data, treatment by time interaction, p < 0.05) (Fig 7.6). By further analysing the Percentage data it was showed that the Ethanol group showed a significant reduction in half-width duration between time periods 1 and 2 of the percentage data (Percentage data, p < 0.05).

Peak-to-Trough

The results from the analysis of Peak-to-Trough show that both Saline and Ethanol groups show a reduction in Peak-to-Trough values over time (Value data, p < 0.05; Percentage data, p < 0.05), however the data is insufficient to state that 80mM ethanol reduces that peak-to-trough values any more than saline alone in the control group (p > 0.05) (Fig 7.7). On the other hand, post-hoc analysis revealed significant differences between time periods 3 and 4 in the Ethanol group, where the Peak-to-Trough value rapidly declines (p < 0.05).
Figure 7.5. The effect of constant ethanol application on the CGC’s frequency. (Left) Shows the change in frequency throughout the experiment in averaged 5 minute time bins. Both groups display a reduction in frequency over time ($F_{2,190}, 24.089 = 25.673, p < 0.05$). Importantly the reduction in the Ethanol group display a greater reduction in frequency over time than the Saline group (Group by time interaction, $F_{2,190}, 24.089 = 5.109, p < 0.05$). (Right) Shows the change in frequency as a change in percentage over time, where the time bins are further grouped. As above, both groups show a reduction in frequency ($F_{1,760}, 19.355 = 27.028, p < 0.05$) with the Saline group dropping to $\sim$75% of baseline and the ethanol treated group, to $\sim$40% indicating a between group significance (Group by time interaction $F_{1,760}, 19.355 = 5.483, p < 0.05$). Error bars indicate $\pm$SEM.
Figure 7.6. The effects of constant ethanol application on the CGC’s Half-width. (Left) Shows the change in half-width throughout the experiment in averaged 5 minute time bins. Both groups show a reduction in half-width over time (Main effect of time, $F_{1.522, 12.175} = 17.484$, $p < 0.05$). (Right) Shows the change in amplitude as a change in percentage over time, where the time bins are further grouped. As above, both groups display a reduction in half-width (Main effect of time. $F_{4, 49} = 33.410$, $p < 0.05$). However, in this data it was revealed that ethanol treatment significantly reduced half-width more than saline treatment (Treatment by time interaction, $F_{4, 49} = 2.646$, $p < 0.05$). It was also found that the reduction within the Ethanol group alone was significant between time periods 1 and 2 ($T_{s} = 4.536$, $p < 0.05$). Error bars indicate ±SEM.
Figure 7.7. The effects of constant ethanol application on the CGC’s Peak-to-Trough duration - the duration it takes for a spike to go from peak to maximal hyperpolarisation. (Left) Shows the change in Peak-to-Trough throughout the experiment in averaged 5 minute time bins. Both groups show a reduction in Peak-to-Trough over time (Main effect of time, F(1.445, 15.898) = 21.490, p < 0.05). However, there was no group by time interaction (F(1.445, 15.898) = 2.445, p > 0.05). (Right) Shows the change in Peak-to-Trough as a change in percentage over time, where the time bins are further grouped. Both groups show a reduction in Peak-to-Trough duration (Main effect of time, F(1.199, 13.185) = 27.655, p < 0.05). Additionally t-tests revealed a significant within-group differences in the 3rd and 4th time periods (3rd time period, T5 = 6.291, p < 0.05; 4th time period, T5 = 6.654, p < 0.05). Error bars indicate ±SEM.
Repolarisation

Results from the repolarisation duration analysis indicated no effect of time, nor any effect of 80mM ethanol treatment (Value data, $p > 0.05$; Percentage data, $p > 0.05$). There is little difference between groups and little change within groups (Fig 7.8).

7.3.1.3 Interim Conclusions

To conclude, a constant ethanol application had no effect on the Amplitude or Repolarisation but did have significant effects on the Frequency, Half-Width and Peak-to-Trough duration values of the CGC spiking characteristics.

However, constant ethanol application does not take into account the variability of ethanol concentrations seen in drinkers and in animal experiments using periodic ethanol applications (‘binge drinking models’) and does not address the question of what would happen if ethanol was removed from the system. Would these affected characteristics be returned to their normal values? This question was addressed by using an intermittent in vitro ethanol application protocol in Lymnaea.
Figure 7.8. The effects of constant ethanol application on the CGC's Repolarisation – the duration from maximal hyperpolarisation back to resting membrane potential. (Left) Shows the change in Repolarisation throughout the experiment in averaged 5 minute time bins. Both show subtle yet statistically insignificant increases in repolarisation duration (Main effect of time, F2.228, 24.504 = 1.755, p > 0.05; Group by time interaction, F2.228, 24.504 = 0.722, p > 0.05). (Right) Shows the change in Repolarisation as a change in percentage over time, where the time bins are further grouped. As above there was no significant differences between or within groups (Main effect of time, F1.840, 20.245 = 2.093, p > 0.05; Group by time interaction, F1.840, 20.245 = 0.592, p > 0.05). Error bars indicate ±SEM.
7.3.2 Experiment 2: The effects of intermittent ethanol application on the CGC

7.3.2.1 Specific Methods

By using an intermittent ethanol protocol it was aimed to determine how the effects of constantly applied 80mM ethanol differ from those observed with alternating periods of presence and absence of ethanol. Also wanted to learn if there is a critical time duration for ethanol application to cause an effect.

Baseline was recorded without perfusion of ethanol for the first 10 minutes. This was followed by a 15 minute of 80mM ethanol perfusion, which in turn is followed by a 15 minute saline perfusion. These latter two stages are repeated once more to reach a total recording time of 70 minutes (Fig 7.1).

7.3.2.2 Results

Amplitude

In this experiment there was a significant overall time-dependent reduction in amplitude (Value data, p < 0.05), the origin of this being the Intermittent Ethanol group (Value data, p < 0.05) (Fig 7.9). Intermittent Ethanol also caused an overall difference between groups (Value data, p < 0.05), however this data did not yield a group by time interaction (Value data, p < 0.05; Percentage data, p > 0.05). The Percentage data did yield a significant within group change in the Intermittent Ethanol group between time periods 3 and 4 (p < 0.05). See Fig 7.10 for an example of the spike pattern created by the CGC with and without ethanol present.
Figure 7.9. The effects of intermittent ethanol application on the CGC’s amplitude. (Left) Shows the change in amplitude throughout the experiment in averaged 5 minute time bins. Both groups show a reduction in amplitude over time (Main effect of time, $F_{2.744, 49.392} = 5.299, p < 0.05$), with the Ethanol group underlying this difference ($F_{2.155, 19.039} = 4.808, p < 0.05$). There was also an overall difference between the groups (Main effect of group $F_{1, 18} = 4.902, p < 0.05$), however there was no group by time interaction ($F_{2.744, 49.392} = 1.992, p > 0.05$). (Right) Shows the change in amplitude as a change in percentage over time, where the time bins are further grouped. The results were not significant enough to yield a difference between groups ($F_{2.174, 39.125} = 2.091, p > 0.05$). Intermittent Ethanol caused a significant change to occur between the 3rd and 4th time periods ($T_9 = 5.759, p < 0.05$) indicated by an asterisk. Error bars indicate ±SEM.
Figure 7.10. Examples of the difference seen in spike images after intermittent ethanol treatment. (A) Control during the baseline period. (B) Control towards the end of a recording. (C) Intermittent ethanol during the baseline period. (D) Intermittent ethanol at the end of a recording.
Frequency
Both the Saline and Intermittent Ethanol groups experienced a decline in frequency at similar rates (Value data, p < 0.05; Percentage data, p < 0.05) and also show a difference between groups (Value data, p < 0.05). However, there was no group by time interaction (Value data, p > 0.05; Percentage data, p > 0.05) (Fig 7.11). By using post-hoc paired t-tests it was revealed that ethanol significantly reduces frequency between the baseline and period 1 (Percentage data, p < 0.05), and between periods 3 and 4 (Percentage data, p < 0.05). However the Saline group also showed similar within group changes albeit at slightly different time periods. Periods 1 and 2 (Percentage data, p < 0.05), and periods 3 and 4 (Percentage data, p < 0.05).

Half-Width
Both groups show a decline in Half-Width duration with respect to time (Value data, p < 0.05; Percentage data, p < 0.05), but not a group by time interaction (Value data, p > 0.05; Percentage data, p > 0.05) (Fig 7.12). However, post-hoc t-tests revealed that the Intermittent Ethanol group was the only group to show within-group changes between grouped periods 1 and 2 (Percentage data, p < 0.05) and periods 2 and 3 (Percentage data, p < 0.05).

Peak-to-Trough
Both groups show a reduction in Peak-to-Trough over time (Value data, p < 0.05; Percentage date, p < 0.05) (Fig 7.13). However, further analysis indicated that both group displayed this effect on their own and there was no indication that 80mM intermittent ethanol affected Peak-to-Trough any differently than saline (Value data, p > 0.05, Percentage data, p > 0.05)

Repolarisation
The intermittent ethanol repolarisation data was insufficient to reveal any significant differences (p > 0.05) (Fig 7.14). Both groups displayed similar repolarisation values and neither group showed any differential change in repolarisation duration over time (Value data, p > 0.05, Percentage data, p > 0.05).
Figure 7.11. The effect of intermittent ethanol on the CGC’s frequency. (Left) Shows the change in frequency throughout the experiment in averaged 5 minute time bins. Both groups show declines in frequency and at similar rates (Main effect of time, $F_{3,591, 64,636} = 37.556$, $p < 0.05$) and they also differ between groups (Main effect of group $F_{1, 18} = 4.644$, $p < 0.05$). However, there is no group by time interaction ($F_{3,591, 64,636} = 0.82$, $p > 0.05$) (need the stats). (Right) Shows the change in frequency as a change in percentage over time, where the time bins are further grouped. Again both groups show reductions in frequency (Main effect of time, $F_{2,633, 47,394} = 68.153$, $p < 0.05$). However, there was no group by time interaction ($F_{1,879, 33,819} = 0.966$, $p > 0.05$). The Intermittent Ethanol group showed significant within group changes between baseline and the 1st period (Percentage data; $T_9 = 3.962$, $p < 0.05$), and periods 3 and 4 ($T_9 = 3.499$, $p < 0.05$). Whereas the Saline group showed differences between periods 1 and 2 ($T_9 = 6.086$, $p < 0.05$), and 3 and 4 ($T_9 = 3.672$, $p < 0.05$). Error bars indicate ±SEM.
Figure 7.12. The effects of intermittent ethanol on the CGC’s Half-width. (Left) Shows the change in Half-width throughout the experiment in averaged 5 minute time bins. Both groups experienced reductions in Half-Width duration (Main effect of time, $F_{3,059} = 9.441, p < 0.05$). However there was no significant group by time interaction ($F_{3,059} = 0.482, p > 0.05$). (Right) Shows the change in Half-Width as a change in percentage over time, where the time bins are further grouped. Again, both groups show a reduction in Half-Width duration (Main effect of time, $F_{2.416} = 10.733, p < 0.05$), but no significant group by time interaction ($F_{1.664} = 0.423, p > 0.05$). Post-hoc analysis of the Intermittent Ethanol group showed significant decreases between periods 1 and 2 ($T_9 = 3.283, p < 0.05$), and 2 and 3 ($T_9 = 3.562$). Error bars indicate ±SEM.
Figure 7.13. The effects of intermittent ethanol on the CGC’s Peak-to-Trough. (Left) Shows the change in Peak-to-Trough throughout the experiment in averaged 5 minute time bins. Together both groups show a reduction in Peak-to-Trough duration ($F_{2,956, 53.200} = 3.084, p < 0.05$). With no interaction between the groups ($F_{2,956, 53.200} = 1.048, p > 0.05$). (Right) Shows the change in Peak-to-Trough as a change in percentage over time, where the time bins are further grouped. This data also revealed a reduction in Peak-to-Trough duration percentage ($F_{2.048, 36.866} = 4.233, p < 0.05$), but no between group interaction ($F_{2.048, 36.866} = 0.882$). Error bars indicate ±SEM.
Figure 7.14. The effects of intermittent ethanol on the CGC's Repolarisation. (Left) Shows the change in amplitude throughout the experiment in averaged 5 minute time bins. Both groups show similar levels of repolarisation, with little variation (Main effect $F_{3.418, 61.532} = 1.323$, $p > 0.05$; Group by time interaction $F_{3.418, 61.532} = 1.081$, $p > 0.05$). (Right) Shows the change in amplitude as a change in percentage over time, where the time bins are further grouped. When expressed in this format, small variations can be noticed in the ethanol group, which may represent an ethanol related effect. However, there is no statistical confirmation of this (Main effect $F_{2.191, 39.440} = 1.200$, $p > 0.05$; Group by time interaction $F_{2.191, 39.440} = 0.423$, $p > 0.05$). Error bars indicate ±SEM.
To conclude, there was a significant effect of intermittent ethanol on amplitude, showing greater reductions in amplitude than controls. Although frequency did reduce over the course of the experiment the Saline group also showed this change and therefore it was not possible to conclude that this was caused by ethanol like in the first experiment. Half-Width, Peak-to-Trough and Repolarisation did not show any significant changes between treatment groups.


7.4 Discussion

7.4.1 Intermittent ethanol application reduces CGC spike amplitude whereas constant ethanol application does not

Notably, there are different effects on amplitude between the two ethanol protocols. Constant ethanol application resulted in very little change in amplitude and matched the result of their control group. However, when ethanol is applied in an intermittent protocol there is a reduction in amplitude. This reduction only starts once ethanol is removed from the system and the amplitude declines during the saline application period. Once ethanol is perfused for the second period, the decline in amplitude ceases, but the pre-ethanol amplitude level does not recover. During the final saline period again, there was a reduction in amplitude. The rate of the decline is equal in both saline periods and also both ethanol periods.

It is not understood what causes this decline in amplitude as a result of the removal of ethanol, however speculation could suggest that the CGC becomes acutely adapted to ethanol, and when it is present the amplitude remains stable, as in the constant ethanol experiment and likewise in the constant saline controls.

The reduction in spike amplitude as a result of the intermittent ethanol procedure could affect the CGC’s communication with other cells and impair its function in associative memory due to a reduction in transmitter release from the presynaptic terminals of its axonal side-branch in the cerebral neuropile that has been shown to play a key role in mediating the effects of its learning-induced somal depolarization (Nikitin et al., 2013, Kemenes et al., 2006b).

It could be suggested that the switching of perfusion fluids may affect the CGC, and could be the reason for the difference seen in the intermittent ethanol group. However the saline group also underwent fluid changes in the same fashion as the saline-ethanol wash outs, and in this group there is little change, therefore it is possible to assume that the physical action of removing a solution is not sufficient to reduce the cell’s spike amplitude.
7.4.2 A constant supply, long duration application of ethanol reduces the spiking frequency of the CGC

When ethanol is applied constantly for 80 minutes reduces the firing frequency of the CGC and results in the frequency being reduced to around 40% and controls to around 75% of their respective baseline. When ethanol is applied using an Intermittent protocol, the decrease in frequency is less, although still present. However, due to the unexpected reduction of frequency in the Control group in the intermittent ethanol experiment, the results were insignificant to conclude that intermittent ethanol group differed from saline treated controls. These findings support other findings such as in *Helix aspersa*, where 50mM ethanol suppressed spontaneous action potentials (Cooper, 2005), as 80mM ethanol also suppressed the tonic firing pattern of the CGC.

When comparing between experiments, it is noticeable that the longer the duration of ethanol the greater the reduction of the firing frequency. There was no suggestion that removing ethanol for periods in the intermittent experiment allows for recovery or slowing of the effect on frequency, and the decline remained linear.

The frequency variable in this study is relatively independent of the other variables, and as such can be an important indicator of the effects of ethanol. The tonic firing activity of the CGC is critical to its function, and disruptions of its consistent firing frequency may affect its role as a modulatory neuron in learning and memory, likely the larger the change from its baseline causing the most change.

7.4.3 A constant but not intermittent ethanol procedure reduces half-width duration of the CGC spike

When ethanol was applied constantly for 80 minutes there is a large reduction in half-width to around 40% of its baseline value, whereas when ethanol is applied in the intermittent protocol the reduction in half-width is to only 70% of baseline, matching the reduction seen in both experiments’ control groups.

The difference between the two procedures may have resulted from either an increased total ethanol duration in the constant ethanol group, or from an irreversible effect of the
initial application of ethanol or perhaps the first 20 minutes of ethanol application was not long enough to start the reduction in half-width. The latter appears to be most likely as the reduction in half-width in the constant ethanol procedure reduces sharply during the first two periods; using this comparison, the intermittent procedure would have had saline perfused during the second period, ethanol may have reached a threshold during this second period.

7.4.4 Constant, but not intermittent ethanol reduces the Peak-to-Trough duration of the CGC spike

A constant supply of ethanol reduced the peak-to-trough duration when compared to control values, whereas intermittent ethanol application showed well matched peak-to-trough durations with controls. The effect of ethanol on this variable shares characteristics with half-width; in that the impact of ethanol appears to have occurred after period 2, and that a threshold has been reached causing this reduction.

7.4.5 Ethanol had no effect on Repolarisation duration of the CGC spike

Ethanol had no direct effect on repolarisation in any procedure that was used. There is an unusual effect seen in the constant ethanol application (Fig 7.8 and 7.14), when up to the second period the repolarisation appears to show an increase, it is during these periods that other variables appear to be affected by the constant ethanol procedure. However, by the end of the values were well matched with controls. This may reflect a ceiling value, and with the application of ethanol the ceiling value has been reached earlier.

7.4.6 Amplitude, peak-to-trough and half-width share characteristics

There is dependence between the three variables; amplitude, peak-to-trough and half width. The way in which these variables are calculated are similar, and in some cases dependent on each other. For instance, a reduction in amplitude may cause a reduction in half width, and may also cause a decrease in peak-to-trough as the peak will be at a less positive value.
Interestingly there was a reduction in the half width and peak-to-trough in the constant ethanol experiment without any effect on amplitude levels. Together with studies implicating the A-type K⁺ current as a target for very high concentrations of ethanol on neurons (Alekseev et al., 1997, Treistman and Wilson, 1987), and characterising the currents of the CGC (Staras et al., 2002) it could be proposed that ethanol is acting on A-type K⁺ channels. Moreover, in the experiments ethanol appeared to need to have a critical duration to initialise its impairment of frequency peak-to-trough and half width. In the intermittent procedure this duration was cut short and once the ethanol was re-applied the effect of ethanol did not appear to be additive. Other studies have shown that the effect of ethanol on the A-type K⁺ current are reversible on washout (Alekseev et al., 1997, Treistman and Wilson, 1987), further supporting the hypothesis that effects of 80mM ethanol on the CGC is, in some way down to its effect on the A-type K⁺ current of the CGC.

### 7.4.7 Conclusions

To conclude, when a constant supply of 80mM ethanol is perfused over the Lymnaea CNS it reduces the firing frequency, half width and peak-to-trough durations of the CGC’s action potentials. However if the same concentration of ethanol is replaced by a saline solution every 15 minutes so that the application protocol becomes more intermittent in nature, then the firing frequency is less affected when compared to controls. Moreover the half width and peak-to-trough durations are both consistent with controls. These findings therefore demonstrate important differences in the effects of constant versus intermittent protocols. However, during the saline periods of only the intermittent ethanol procedure was the amplitude reduced, and when ethanol was reapplied the reduction in amplitude stopped. These differences between how even different protocols of ethanol application interact with the basic electrical functioning of the same identified neuron are a major new finding from this study. Additional work will be needed to elucidate the changes in ionic currents that underlie the electrical changes observed in this study.
Chapter 8 – General Discussion

This thesis has presented evidence for alterations in learning and memory mechanisms following the application of high ethanol concentrations in a number of different experimental protocols, and in two different animal models. Due to the diversity of the experiments and their results first it will be useful to outline their conclusions before offering a general discussion of the findings.

**The effects of AIE treatment on spatial memory in mice:** It was found that AIE treatment did not affect spatial memory in a MWM task, when tested without ethanol being present. However, when ethanol was present spatial memory was impaired. AIE did not affect acquisition of an object recognition task, but did facilitate a spatial memory task without affecting a non-spatial memory task. In a test of locomotor activity, mice acutely treated with ethanol showed higher scores in the first five minutes of a 60 minute test session but lower scores across the entire test period.

**The effects of AIE treatment and ethanol on hippocampal LTP in mice:** 60mM ethanol was found to inhibit hippocampal LTP when it was present during high frequency stimulation (HFS), or when it was applied to the slice for 30 minutes prior to HFS with a rapid increase in ethanol concentration. However, if the ethanol concentration was raised slowly LTP could be induced. Adolescent intermittent ethanol (AIE) treatment created a NMDA-dependent slow transient increase in fEPSP slope% over time, even in the absence of LTP.

**The effects of ethanol treatment on operant conditioning in mice:** Post-trial 2g/kg injections of ethanol impaired lever pressing for reward performance, whilst not indicating a strong aversion to 2g/kg ethanol in a conditioned taste aversion (CTA) experiment. However, because the experiment investigating possible induction of a CTA did not complete replicate the ethanol administration protocol used in the repeated injection model for the operant conditioning task, it was not possible to completely exclude the possibility that reduced operant responding reflected the induction of a CTA.

**The effects of ethanol on associative learning in Lymnaea:** Ethanol blocked acquisition and retrieval of an associative memory. However, this effect was found to be state-dependent and if the original condition was matched (i.e. ethanol present at both acquisition and retrieval), then successful retrieval was possible.
The effects of ethanol on a single invertebrate neuron in *Lymnaea*: a constant supply of 80mM ethanol reduced the firing frequency, half width and peak-to-trough durations of the CGC’s action potentials. Intermittent ethanol did not result in the reduction of frequency, nor did it reduce the half-width duration. However, intermittent but not constant ethanol did reduce the amplitude of the CGC spikes.

8.1 Comparing the effects of ethanol on spatial memory and hippocampal LTP

The AIE EtOH-acq group was used to investigate what the effects would be if AIE treatment occurred during the same period of learning (section 3.3.2). This group received ethanol injection 30 minutes prior to performing in the pool, this would therefore lead to a rapid escalation of BEC that could be comparable to the ethanol protocol experienced in the Off-On group and Burst groups that blocked LTP (section 4.3.1). This would suggest that injection of 2g/kg ethanol may inhibit LTP and therefore impair spatial learning, and this could be why the AIE EtOH-acq group did not perform well in the MWM. Although the ethanol injection would cause a rapid rise in BEC it is unlikely that it would have occurred as rapidly as it did in the hippocampal slices of the Burst and Off-On groups, as this was almost immediate. It could be that it occurred more like the Pyramid protocol, which was designed to model a more realistic ethanol escalation. Interestingly this treatment group was able to induce hippocampal LTP.

The difficulty is characterising the effects of the AIE treatment in with regards to hippocampal slice work, what timescale is most suitable? There is good reason to argue that after the injection of ethanol the mice could better reflect the Pyramid group as the onset of BEC could not be as rapid as the all immediate onset seen in the Off-On or Burst groups. However, the AIE EtOH-acq group is probably in-between the two onset timeframes (i.e. between Pyramid group and the Burst and Off-On groups). Therefore, if in this example an injection is more similar to a Burst or Off-On protocol, the types of treatment that block LTP, then this could be a factor in why poor learning was seen in the AIE EtOH-acq group, when compared to other groups. But as these animals were able to learn slowly, perhaps they were not fully affected by this type of treatment.
So how then could this memory have been spared? Is there a way that the AIE EtOH-acq group could have shown better performance in the MWM task? One possibility is that if another model of binge drinking had been used, for example the ‘drinking in the dark’ procedure where ethanol was freely consumed rather than injected, the change in BEC would have been slower, and may have better reflected the Pyramid group. In this circumstance a better performance may have been seen in the MWM in the AIE EtOH-acq group because hippocampal LTP may have not been inhibited and/or it is possible that a switch in the mechanism of LTP may have occurred, resulting in less impairment of spatial memory.

One reason why the MWM may not have picked up on binge drinking induced deficits was the difficulty of the MWM. The difficulty of the maze was increased by reducing the number of trials (which may have created a bias in the entry position) although the mice still acquired this task easily. Another method by which the difficulty could have been increased would be to have used a larger pool. This would have decreased the chance the animals would have been able to locate the platform by chance, therefore they would have to engage their spatial memory more.

8.2 The effects of AIE treatment on memory

In two behavioural and three electrophysiology experiments AIE treatment was performed in C57BL/6J mice. These mice were then tested for the effects the treatment had on memory and hippocampal LTP in adulthood. One of the behavioural tasks was a MWM with reduced training trials to make the acquisition of the task more difficult. It was hypothesised that this would allow the task to reveal even small deficits in memory as a result of AIE treatment. However, there was no evidence to suggest that AIE treatment resulted in a deficit in spatial memory in adulthood. Moreover, during the probe trials both the spatial and non-spatial techniques that the animals could use to correctly navigate the pool were equally effective in locating the escape platform. Also AIE treatment did not result in spatial memory deficits in a spatial memory version of the object recognition task. Quite the contrary, AIE treatment appeared to facilitate spatial memory. AIE may have also affected learning of an operant task, where AIE treatment successfully reduced subsequent lever pressing for a reward. This could have been due to an inhibition of memory at some stage or possibly contextual and may be explained by other means. In the 3 electrophysiology experiments that were performed on mice that had undergone AIE it was clear that AIE had had some effect on the functioning of the hippocampal cells,
as it had produced a slow rising, NMDA-dependent EPSP slope% increase on top of traditional LTP. This too does not support the hypothesis that AIE treatment during adolescence impairs spatial memory in C57BL/6J mice (Weissenborn and Duka, 2003, Townshend and Duka, 2005, Tapert et al., 2004b, Spear, 2015).

So what is happening? Why does the AIE protocol used in this thesis lead to no deficits in spatial memory or LTP, and in some cases improve LTP and spatial memory? Why does this differ from the general consensus of human lead binge drinking research that suggests binge drinking does lead to deficits in spatial memory (Weissenborn and Duka, 2003, Townshend and Duka, 2005, Tapert et al., 2004b, Spear, 2015).

One possible explanation for the increase in EPSP slope% is that the NMDA receptors themselves are being upregulated during the periods of AIE treatment (adolescence). Different NMDA receptor subunits have been shown to be expressed during development with varying sensitivities to ethanol (Hefner and Holmes, 2007). Therefore it could be possible that AIE treatment or binge drinking itself could impose an upregulation of the ethanol sensitive NMDA receptors, resulting in an overall increase to sensitivity in later life. For instance, ethanol is less potent towards NR1-NR2C or NR2D currents than NR1-NR2A or NR2B currents (Hefner and Holmes, 2007). Ethanol may also affect different subtypes of NMDA receptor differentially during adolescent development, and possibly overexpress an ethanol-sensitive form of NMDA receptor over ethanol-insensitive (Hefner and Holmes, 2007). The idea of NMDA upregulation is not a novel idea, general NMDA receptor upregulation is seen in human alcoholics and models of chronic alcohol use alike (Xia et al., 2006, Wang et al., 2012), this upregulation can result in over-activation and seizure activity. If this were true for the AIE treatment, it would be expected to be of less severity than alcoholism or chronic ethanol treatments.

An example of this seizure activity may have been observed in chapter 4, where a NMDA-dependent transient increase in EPSP slope% was established in all AIE treated animals on top of traditional LTP. When NMDA receptors were not blocked the output continually grew, this may be as a result of increased susceptibility to seizure activity.

Another possibility is that AIE is causing an increased ethanol sensitivity to the tonic current in adult dentate gyrus granule cells, in a similar way to the work performed by Fleming et al (Fleming et al., 2013). It is thought that this action could explain their previous work that showed increased sensitivity to the memory-impairing effects of
ethanol in adulthood after previous adolescent exposure (White and Best, 2000, Fleming et al., 2013).

It is known that binge drinking results in hippocampal damage (Medina et al., 2007, De Bellis et al., 2000), and in hippocampal neurodegeneration (Crews et al., 2004). As a result of this damage the hippocampal slices of AIE treated animals may be more sensitive to the disruptive effects of being removed from the organism and undergoing hippocampal slicing. For example, there may be an increase in glutamate leakage resulting in increases fEPSP’s (Fleming et al., 2013). Another possibility is that the GABA recurrent inhibition of the pyramidal cells are affected in the AIE treated hippocampi, a reduction in the inhibition of the pyramidal cells could lead to an increase in fEPSP (Izumi et al., 2007, Zorumski et al., 2014).

It is important to note that memory is not confined to the hippocampus and there are many of neural regions involved in memory that have also been shown to be impaired by ethanol treatments (Zorumski et al., 2014). For instance in the amygdala synaptic plasticity plays a crucial role in fear conditioning, a well-known model of emotional learning (Ehrlich et al., 2009). Furthermore the amygdala makes many connection to and from the hippocampus, and this is thought to develop context to fear conditioning (Zorumski et al., 2014). In the lateral amygdala LTP is associated with fear conditioning and when expressed it increases behavioural response to fear stimuli (Johansen et al., 2011). Like hippocampal LTP lateral amygdala LTP is initiated by NMDA receptors and is expressed by the insertion of synaptic AMPA receptors and increased efficacy of transmission (Johansen et al., 2011, McCool, 2011). Both acute and chronic ethanol treatments have been shown to dampen LTP in the amygdala, and that this is thought to occur primarily via NMDA receptor antagonism, and partial via increased GABAergic inhibition (Roberto et al., 2012). Acute ethanol also dampens LTP in the dorsal striatum and even convert LTP into LTD if the ethanol concentration is above 50mM (Yin et al., 2007). Other studies have shown that acute ethanol can even dampen MAPK pathways within striatal regions (Xie et al., 2009). Intermittent ethanol treatments have been demonstrated to upregulate expression of GluN2B showing facilitated LTP in the striatum (Xia et al., 2006, Wang et al., 2012). Perhaps the effects of ethanol on these regions had some impact on the reduction in performance of the ethanol treated mice in the operant chambers. The cerebellum is also known to be effected by ethanol. The cerebellum is involved in motor control and coordination and is also the region for motor learning. Ethanol acts on the cerebellum to cause motor incoordination and long-term alcoholism
can result in persistent dysfunction (Valenzuela et al., 2010). Both climbing fibre and parallel fibre LTD are both blocked by 50mM ethanol, this too is thought to be mediated via GluR1 and Ca$^{2+}$ channels (Belmeguenai et al., 2008, Su et al., 2010).

### 8.3 Criticisms of animal models of adolescent binge drinking

Though no mammalian model can perfectly model for a human disorder it does allow for a highly controlled environment and procedures, allowing the researcher to accurately know how much ethanol the subject has consumed. Certain methods of ethanol administration are better suited to model for different situations. For instance oral administration may better reflect drinking but it is difficult to control the BAC when compared to gavage or i.p. For instance gavage and other intragastric ethanol procedures can result in very high doses of orally administered ethanol. However, this probably doesn’t reflect human ethanol consumption (Sprow and Thiele, 2012). Other methods restrict access to water in order to increase the desire to consume liquids later when presented with an ethanol solution. This is unnatural and stressful, and again is unlikely to occur in healthy humans (Sprow and Thiele, 2012). Nonetheless, rodents make excellent models for most of the effects that alcohol may lead to.

One of the problems that arose when performing the AIE treatment in mice is the limited duration that mice are in adolescent development. Due to the limited duration that rodents are considered adolescent PND 30 – 60 (PND 45 is considered late adolescence) (Pascual et al., 2007, Van Skike et al., 2012, Varlinskaya et al., 2001), it is difficult to perform enough ethanol treatments to truly replicate human binge drinking. Human binge drinking appears to have its cognitive implications in females after around 60 days (Loeber et al., 2009, Weissenborn and Duka, 2003). Which is considerably more episodes of ethanol than it is possible to produce in mice without resulting in ethanol-dependence (Pascual et al., 2007, Pascual et al., 2009, Ripley and Stephens, 2011). And indeed most studies use alcohol preferring rodents. Rodents who actively avoid ethanol show different results to ethanol preferring rodents when treated with ethanol (Ripley and Stephens, 2011, Oliver et al., 2009). With this in mind, one would assume that the majority of human binge drinkers are going to be ‘alcohol preferring humans’, so perhaps this is not a major issue and isn’t just for the ease of experimentation. What would be interesting is that if ‘alcohol avoiding humans’ binge drink are they differentially
affect in the same manner as alcohol avoiding rodents? Although this is beyond the scope of this thesis.

It is also very difficult to test for higher order deficits in mammalian models, and tests of executive functioning is often hard to establish (Acheson et al., 2013). Few animal studies have actually displayed that chronic and AIE treatments have led to higher order deficits when compared to humans (Obernier et al., 2002b, Kroener et al., 2012). Moreover, human binge drinking has many associations that are difficult to model in animals (Sprow and Thiele, 2012, Spear, 2015, Doremus et al., 2005). Although it is of some considerable benefit to simplify behaviours to learn about them, this may cause some bias in the results.

Binge drinking typically occurs in a social setting, how much this social setting impacts on how ethanol affects the brain is complicated (Petit et al., 2014, Stickley et al., 2013, Field et al., 2008). One example of the unusual effects ‘social settings’ is that when humans, in a group are given ethanol-free ‘alcoholic’ drinks, they act in a way which is considered intoxicated (Assefi and Garry, 2003). It is unlikely that this can occur in rodent models, which are typically single or pair housed. Binge drinking is much more than the consumption of ethanol. Behaviours like social interaction, dancing, interrupted/poor sleep, and sexual interaction are all common with binge drinkers. All of these behaviours are able to have a profound effect on the brains neurochemistry, as well as being extremely difficult to control for.

There has also been suggestion that when treating young people for depression, targeting binge drinking may help prevent neurobiological change that may underlie the poor clinical results (Hermens et al., 2013). Findings such as this are generally not considered when modelling human adolescent binge drinking. Perhaps one reason why differences are seen between human binge drinkers and animal models of binge drinking is that depression is motivating for binge drinking and this co-expression could result in some of the cognitive deficits seen in humans. For instance, AIE treatments in rats have shown increased expression of corticotropin in the hypothalamus (Przybycien-Szymanska et al., 2011), decreased levels of neuropeptide Y in the hippocampus and increased substance P and neurokinin IR in the caudate (Slawecki et al., 2005, Lerma-Cabrera et al., 2013).
8.4 Could binge drinking during adolescence cause harm to later life memory?

One of the original hypothesis set about by this thesis was the idea that binge drinking during late adolescence could cause harm that would continue later in life. The AIE treatments used in this thesis showed no behavioural deficits in spatial memory (Tapert et al., 2004b, Townshend and Duka, 2005). However, AIE treatment did show an unusual, possibly novel form of LTP in the hippocampal slice experiments. There is growing evidence that although ethanol may not always obviously impair a function or memory, it could be modulating it by inducing by a different mechanism, which may or may not be harmful (Izumi et al., 2015, Tokuda et al., 2013, Zorumski et al., 2014, Sabeti, 2011, Sabeti and Gruol, 2008).

A generally accepted principle within alcohol research and society in general is that drinking low percentage alcoholic drinks in a slow and controlled manner is better for your health than drinking the same amount of alcohol in a short burst. This may be culturally obvious, but the reasons why were not so. The work performed on hippocampal slices aimed to better reflect real life BAC’s resulting from binge drinking, and by doing so it was shown that LTP could be induced if the ethanol concentration was raised and reduced slowly, but not induced if the concentration was raised slowly and maintained, although another study has shown LTP in this circumstance (Tokuda et al., 2007).

If this type of rise in ethanol concentration does better model binge drinking then it would suggest that binge drinking may result in an altered LTP mechanism. In some ways this could be compared to an alcohol induced memory blackout where encoding is poor, and on the following days there is little to no retrieval of the previous day. It has been shown that when the rise and fall in ethanol concentration was gradual LTP could be induced, therefore it could be suggested that if a drinker slows the rate at which they consume alcohol, they are less likely to disrupt or switch LTP mechanisms.
8.5 The shared effects that alcohol has on vertebrates and invertebrates

It is often difficult to directly compare such different species as snails and mice but it is possible to make careful comparisons across the species. For instance, there is a similarity in the effects of fast onset, high concentrations of ethanol. In both models the electrophysiology experiments revealed a reduction in neuronal output, in the CGC this is seen by a reduction in spike frequency (continuous ethanol), and amplitude (intermittent ethanol), and in the hippocampus this could be seen by the blockade of LTP. Therefore in both examples ethanol is able to depress both neuronal systems and possibly reduce the general effectiveness of memory.

If the effects of ethanol on behavioural experimentation were compared between the two models then there is also some noticeable similarities. In the mouse it was found that a 30 minutes prior injection of a high concentration of ethanol before training/testing impaired performance without impairing motor function. In the snail 30 minutes pre-training injection of ethanol too blocked retrieval of memory. It is interesting that the effect and duration are consistent between species, this may suggest that the underlying, highly conserved mechanisms behind these results are shared between the species and likely many others.

There was also some speculation in both models around whether ethanol impaired consolidation of memory. When investigating the effect of ethanol on memory consolidation in the snail, a single post-training injection of ethanol appeared to reduce the strength but still allow the formation of a memory. Similarly, when post-training injections of ethanol were performed in mice, in the operant task, it was noticed that their performance would become repeatedly weaker until the memory appeared to disappear. In the snail model the reduction seen was statistically insignificant. In the mouse the reduction could have been explained by a CTA. It is unlikely that a CTA would have developed in the snail model as the contingency between the conditioning and injection was too far apart.

Given the similarities between the generalised effects of ethanol on snails and mice. It is possible that the snail model could become a useful model to investigate the cellular and molecular impact of ethanol and other such drugs on memory. This would support existing invertebrate models of ethanol use such as Drosophila and C.elegans which are
becoming increasing useful in ethanol-related investigations. This would also progress towards the 3R’s principle (Tannenbaum and Bennett, 2015), something that would be of benefit to science, governments and the general public alike.

8.6 Ethanol alters associative memory in *Lymnaea* and the basic properties of the CGCs

The results from the snail model showed that ethanol inhibits retrieval of an associative memory if injected 30 minutes prior to testing. Importantly, it was found that this effect is state-dependent and if the snail learnt the conditioned association whilst under the effects of ethanol then the snail would be able to retrieve the memory. Numerous experiments were performed looking for a drug that could mimic the retrieval of ethanol learnt associative memory. Although no single drug could replicate the result achieved by ethanol, NMDA did produce an increased response in the ethanol trained snails when compared to saline trained, which is what would be expected if ethanol was to be substituted by another substance.

By performing electrophysiological recordings of the CGC whilst perfusing an ethanol solution over the CNS, a reduction in firing frequency, half width and peak-to-trough durations was shown. These reductions would likely affect the ability of the CGC to continue to function as a modulatory neuron within the memory system. Therefore it could be that reductions in these intrinsic properties are creating the circumstances that are impairing the memory in the behavioural experiments. For example, after classical conditioning the membrane of the CGCs become persistently depolarised, without changing the rate or spike shape of the tonic firing pattern (Vavoulis et al., 2010). If however, the ethanol is applied and does reduce the firing rate, or spike amplitude it is likely to affect this conditioning. Moreover, if these properties were affected before the snails were trained, then the memory would have been acquired and encoded using less frequent, shorter spikes, this different pattern of information may have resulted in a modified plasticity, a similar idea to what has been noted in mouse models of memory (Sabeti, 2011, Sabeti and Gruol, 2008, Izumi et al., 2005, Tokuda et al., 2007, Zorumski et al., 2014). However, when the snail is next tested after an injection of ethanol, the CGC’s unusual firing pattern is the same as it was during acquisition and this could allow for retrieval and could provide an explanation into how the state-dependency was being expressed.
8.7 Comparing the snail’s state dependency with studies of mice

Possibly the most intriguing finding within this thesis was that of ethanol state dependent learning in the snail model. Unfortunately, none of the mouse behavioural studies had a strict state dependent learning paradigm in them. However, the AIE EtOH-acq group in the MWM experiment was repeatedly trained after an injection of ethanol. However this group acquired the task poorly, and although its performances in the saline and ethanol injected probe trials were equal, it is hard to express a difference between treatments if the original acquisition was not strong. If this group was continued to be trained until it had reached a criterion of performance, it may have been possible to notice improvements in the ethanol over saline probe trials. For this experiment to clearly show state-dependent learning the Saline group would have had to have shown an impaired performance in the ethanol probe trial, and this was not the case. Other studies have shown that both 1.75 and 2.25g/kg ethanol injections did impair spatial memory in C57BL/6J mice in the MWM after sufficient training (Berry and Matthews, 2004).

Ethanol state dependent learning does exist in mice, for instance adult male NMRI mice having been injected (i.p.) with 0.5g/kg or 1g/kg before training and testing and this resulted in state dependent retrieval of memory in a passive step-down avoidance task (Rezayof et al., 2007, Rezayof et al., 2008b). This was later concluded to be due to the dorsal hippocampal NMDA receptors, which themselves can be mediated by the hippocampal nitric oxide system (Rezayof et al., 2010b, Rezayof et al., 2008b). In chapter 6 it was speculated that NMDA appeared to be the most likely of the 4 drugs that were investigated in the snail to ‘reproduce’ the ethanol state dependency. Taken together these two results reinforce the speculation that ethanol induced state dependent learning is, in some way, NMDA receptor dependent in Lymnaea.

These experiments could be further developed by adapting the doses, and/or combining more than one drug, since the nature of ethanol is not defined to a single receptor (Deitrich et al., 1989). Due to the clear learnt/not-learnt results in these experiments and its well conserved receptor homology (Ha et al., 2006, Kemenes, 2013), it has the possibility of becoming a worthy model for the investigation of other drugs impact on associative conditioning and memory. An interesting possibility is that one of the drugs used in the pharmacological investigation of the ethanol state dependent behaviour would also be able to produce a reduction in frequency, peak-to-trough and half width in
the CGC if it were perfused over the CNS. This would support the idea that ethanol mediated a change in the mechanisms of memory formation and would need ethanol to recreate that change again before it can be retrieved.

8.8 General Conclusions and Outlook

In contrast to the growing literature regarding the effects of binge drinking during late adolescence, there was not sufficient evidence of impairment in later-life using the mouse model of binge drinking. In some cases spatial memory, and hippocampal LTP were found to be improved in young adulthood after late adolescent binge drinking. However, it is possible that the mechanisms of LTP resulting in these forms of memory are altered as a result of binge drinking. There was evidence however for the impairment of binge drinking on memory if injected either immediately before or after training in a MWM and operant lever pressing task respectively. This study therefore opens up new avenues towards more systematic future studies investigating the time windows after learning during which ethanol may have a deleterious effect on long-term memory.

By utilising the snail model of learning and memory Lymnaea stagnalis, important findings have emerged regarding the effects of high ethanol concentrations on associative memory. Ethanol injection was sufficient to block acquisition and retrieval, as well as reduce the strength of consolidation. Remarkably, it was also shown that the snail could easily perform ethanol-induced state-dependent learning. The CGC, which has a known role in associative memory in Lymnaea, was found to have its firing frequency and spike peak-to-trough and half width reduced when exposed to an 80mM ethanol solution suggesting that ethanol would impair electrical activity of the CGC thus leading to memory impairment. In the past ten years Lymnaea has been used increasingly as a valuable model system for the analysis of the cellular and molecular mechanisms of age-related impairments in feeding behaviour, chemosensory processing, associative memory and neuronal function (Patel et al., 2006, Arundell et al., 2006, Patel et al., 2010, Hermann et al., 2007, Watson et al., 2013, Watson et al., 2012, Watson et al., 2014, Hermann et al., 2014, Pirger et al., 2014) as well as to investigate memory deficits induced by amyloid beta peptides and their underpinning neuronal mechanisms (Ford et al., 2015). The findings presented in this thesis have prepared the way for a further important use for Lymnaea, namely in studies aimed at understanding alcohol-induced memory deficits and the underlying neuronal changes. The development of in vivo and in vitro ethanol treatment and test protocols and the findings
from my experiments based on these protocols open up new avenues for future systematic investigations on ethanol’s effects on behaviour and underlying neural circuitry in *Lymnaea* that could also inform future studies aimed at understanding the effects of ethanol on neuronal circuits and cell types of the mammalian brain involved in learning and memory.
Chapter 9 – References


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