Anaesthesia and Cognition

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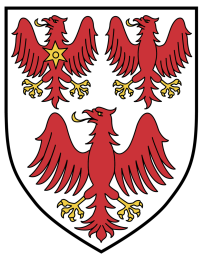
A thesis presented in support of the degree of Doctor of Philosophy



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This thesis is dedicated to the memories of three friends, who cannot be here to see it come to fruition but were part of the journey. You continue to provide inspiration and will always have a place in my heart:

Karla Shar

Young Lee

Corri Waite

Thesis preface

"There is something to be said for the experience of doing a PhD."

Professor David Bannerman during the preparation of
my Wellcome Research Training Fellowship – awarded in October 2008.

If life is a roller coaster, my PhD felt like a representation of the 'Verruckt' in Schlitterbahn Water Parks, Kansas City - a 168 foot water slide coaster whose opening was delayed because sand bags (substituted for people) kept flying out of the side during preparation and safety checks.

The journey began sometime back in 2005 when a very wise mentor (Dr Polly Taylor, a veterinary anaesthetist and now very good friend) said nonchalantly *"You should do a PhD"*. I have always been fascinated by anaesthesia. At 15 years old I found a book – Animal Anaesthesia by C. J. Green – on the shelves of the veterinary practice where I worked part time. I remember that I read it in one evening, in front of the television eating Cadbury's Crème Eggs; and subsequently asked the vet I was working with (Dr Bob Gardner) *"But how does anaesthesia work?"*. His answer was to inspire my career path for evermore… *"Actually, I don't have a clue"*.

My PhD experience has been amazing, and about so much more than my career. I didn't know when I started that it would involve moving to New York City for three years, experiencing all that that had to offer, both personally and professionally. I have been surrounded by superbly talented, intelligent, energetic, generous and supportive people, who have inspired me, enriched my life and kept me going through failed experiments and some tough personal challenges encountered along the way.

I feel as if I've grown from a veterinary surgeon into a veterinary anaesthetist (my dream vocation), and learned more than I imagined possible. Of course the most important thing I've learned is that there is *so* much more to learn. I come away with the words of

Dr Phil Browning ringing loudly in my ears… *"Science is hard!"*. And now have a new level of respect for all who devote time, energy and enthusiasm to the beast that is: scientific advancement.

I would like to thank everyone who has been a part of this journey with me. Those who have supported me both professionally and personally, put up with my 'elastic relationship to deadlines', tolerated my seeming inability to benefit from their voices of experience, been there to pick up the pieces when things didn't go according to plan, and are still there cheering as I approach the finish line… much later than planned.

There isn't enough ink in my printer to thank you all individually but you should know that I am full of gratitude. If you have been given this preface to read, it's because I want you to know that your contribution, no matter how large or small, no matter how direct or indirect, is greatly appreciated (just be thankful that I didn't send you the whole 80,000 word thesis).

Cartoon by Jerome Sallet 2015. Adaptation of 'It's an interesting phenomenon', artist unknown.

Specific acknowledgements

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Examiners:

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In no particular order:

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Dennis

Minnie

Fredi

Izzy

TABLE OF CONTENTS

ABSTRACT

Anaesthesia is an essential component of many clinical and scientific procedures. It is well-established that anaesthesia has short-term effects on cognition but an increasing body of work suggests that anaesthesia may have longer-term effects on learning and memory, beyond the point at which the drugs are eliminated from the body.

Experiments were conducted, in adult rats, to better understand the behavioural phenotype of anaesthetic-induced cognitive impairment. A two-hour exposure to isoflurane/nitrous oxide anaesthesia in the absence of surgery was sufficient to impair win-shift spatial memory on the radial arm maze, despite intact spontaneous spatial novelty preference, a candidate psychological process for radial maze performance. Impairment in attentional processing might also affect spatial memory. The same anaesthetic regime, but not an injectable anaesthetic combination of propofol/fentanyl, impaired some aspects of attention, as assessed with the 5-choice serial-reaction-time task and the sustained attention task (SAT). However, in contrast to the spatial memory impairment, the magnitude of attentional impairment was not increased following successive anaesthetic exposures.

A similar pattern of spatial memory impairment dependent on the number of anaesthetic episodes was seen in rats exposed to isoflurane during development and tested in adulthood. Developmental exposure to a similar anaesthetic, sevoflurane, did not impair attention in adulthood as assessed with the SAT and the intradimensional/extradimensional shift task.

With the aim of better understanding the neuronal mechanisms underlying the spatial memory impairment, laser confocal microscopy was used to examine the dendritic ultrastructure of hippocampal neurons from rats exposed to isoflurane/nitrous oxide during adulthood. Results did not provide compelling evidence for changes in dendritic ultrastructure.

These results highlight that anaesthesia is not benign and is a critical variable that should be considered when designing animal behavioural neuroscience experiments. In addition they contribute to a larger literature regarding the potential long-term effects of anaesthesia on human health.

ABBREVIATIONS

Anatomy, physiology and pharmacology

BOLD	Blood-oxygen-level-dependent
CA1	Cornu Ammonis 1
CO2	Carbon Dioxide
GABA	Gamma-aminobutyric acid
N2O	Nitrous oxide
NMDA	N-methyl-D-aspartate
O2	Molecular oxygen
PCO2	Partial Pressure of CO2
PO2	Partial Pressure of Oxygen

Behavioural testing

IDED	Intradimensional extradimensional shift task
ITI	Inter-trial interval
MWM	Morris Water Maze
RAM	Radial arm maze
SAT	Sustained attention task
SSNP	Spontaneous spatial novelty preference task

Measurement

°C /C	Centigrade
cm	Centimeter
μg	Microgram
μm	Micrometer
ml	Milliliter
mm	Millimeter

Statistical

ANOVA	Analysis of variance
F	F statistic: the ratio of MS treatment to MS error
p	Probability (e.g. $p < 0.05$)
sem	Standard error of the mean

Linguistic

e.g.	For example
et al.	And collaborators
i.e.	Id est (that is)
p*er se*	By or in itself
vs	Versus
vs vs	Vice versa

Other

fMRI	Functional Magnetic resonance imaging
in vivo	with in the living
NaCl	Saline
P	Postnatal day
PBS	Phosphate buffered saline
POCD	Post-operative cognitive dysfunction
UK	United Kingdom
USA	United States of America

CHAPTER 1: GENERAL INTRODUCTION

Origin of thesis

Anaesthesia is an important component of many laboratory animal procedures and is embedded within the legal framework that governs animal research in the United Kingdom (UK) and other countries (for a brief overview of the legal framework, please refer to Appendix 1). This thesis has stemmed from experience working as a clinical laboratory animal veterinarian, with the role of providing advice on the refinement of anaesthetic protocols for animal experiments.

During my clinical work as a laboratory animal veterinarian, it became apparent that limited empirical data exist upon which to provide the necessary advice to researchers, regarding the refinement of anaesthetic techniques and protocols, and potential confounding effects of anaesthesia on scientific results. This realisation came at a time when a question was faced regarding the appropriateness of certain anaesthetic agents for a behavioural neuroscience experiment, and led to a collaborative study (published before this program of work began (Baxter et al., 2008) that formed the impetus for this thesis work. For a detailed description of that study please refer to Appendix 2. The study involved assessing the post-operative behavioural performance of rats that had received identical specific brain lesions under either isoflurane or propofol anaesthesia. Results showed that the pattern of behavioural impairment induced by the brain lesion surgery was dependent on the anaesthetic agent used during generation of that lesion. These results raised questions around the role of anaesthesia as a potential confound for behavioural neuroscience experiments.

This thesis does not follow on *directly* from the above-mentioned study, which showed an interaction between a surgical brain lesion and the anaesthetic agent used, but rather from the experience of investigating the role of anaesthesia as a critical variable in animal experiments. Performance of the sham surgery control groups in the described study did not differ, in other words there was no main effect of anaesthesia. However, as animals in both control groups will have experienced tissue damage, it is possible that any relative effects of anaesthesia are confounded by the inevitable surgery-induced inflammatory response (Cunningham et al., 2009). An increasing body of evidence suggests that anaesthesia could indeed induce post-anaesthetic cognitive impairment when administered in the absence of surgery (Culley et al., 2004b, Jevtovic-Todorovic et al., 2013). To that end, contained here within, is a program of work examining the effects of anaesthesia, *in the absence of surgery*, on post- anaesthetic cognitive function. Experiments were designed to examine the character and expression of the phenotype of anaesthetic-induced cognitive impairment, in healthy rats.

Aims of thesis

The characterisation of anaesthesia (in the absence of surgery) as a confounding factor for neuroscience experiments is the overarching aim of this programme of work. This aim is subdivided into three, more specific, aims.

Aim 1: to better understand the behavioural phenotype of anaesthetic-induced cognitive impairment.

Anaesthetic-induced cognitive impairment could complicate the interpretation of behavioural neuroscience experimental results by decreasing the detection of experimental phenotypes. If control animals (animals that received anaesthesia in the absence of intended experimental manipulation) demonstrated behavioural impairment, between-subjects differences (i.e., the difference in performance between experimental and sham or control animals) would be reduced and may go undetected.

Within-subjects serial comparisons could also be affected. Many laboratory animals undergo repeated anaesthesia (e.g., for imaging procedures, for the placement and removal of optical fibres during experiments involving optogenetics technology, or similarly for the placement and removal of electrode microdrives in electrophysiological experiments). It is not yet widespread practice to include anaesthesia-only control groups. If anaesthesia itself led to changes in the model throughout the experiment (over anaesthetic episodes), this would lead to within-subjects differences that could be mistakenly attributed to other factors, or obscure experimental effects. Experiments that specifically investigate these potential effects

would provide researchers with the evidence base needed to determine the appropriateness of control groups.

Understanding which cognitive domains are involved in anaesthetic-induced cognitive impairment is fundamental to the facilitation of data interpretation, to the ability to appropriately modify experimental design for behavioural neuroscience experiments, and to the identification of the neurobiological substrates of anaesthetic-induced cognitive impairment.

Aim 2: to better understand the conditions that determine the magnitude of effect or expression of the phenotype of anaesthetic-induced cognitive impairment.

Such characterisation is crucial for building an evidence base that can be used by researchers, veterinarians and ethical review committees for the design and interpretation of experiments. Specifically, understanding how to treat control groups would enable account to be taken of the long-term off target effects of anaesthesia.

As described above, one variable in behavioural neuroscience experiments involving anaesthesia is the number of episodes of anaesthesia that animals undergo. Repeated episodes of anaesthesia are often needed to provide humane restraint for laboratory animals used in neuroscience studies, for example to facilitate imaging or the placement of lasers or cannulae. Such within-subjects comparisons (where performance is compared at different time points in the same subject) become more commonplace as legislative controls encourage a reduction in animal numbers *and* refinement of procedures leads to decreased morbidity and mortality, thereby

facilitating the continued use of subjects. Knowing whether anaesthesia may have some cumulative effect, that whilst not impacting on morbidity or mortality may interfere with the scientific outcomes, therefore becomes important. In addition, some countries allow the re-use of animals (i.e., the use of an animal on the same or a different protocol, after completion of one series of regulated procedures, where a previously unused animal would have equally sufficed). Understanding any long-term or cumulative effects of prior anaesthetic exposure is therefore essential, for ensuring that participation in the previous experiment will not affect the results of subsequent experiments.

Another experimental condition that could affect the expression of anaesthetic-induced cognitive impairment is the choice of anaesthetic agents. A wide variety of anaesthetic agents are used in laboratory animal anaesthesia. In addition to choosing agents that minimise physiological derangement (as for clinical anaesthesia), laboratory animal anaesthesia requires that agents are chosen that have the least confounding effects on the scientific output. If the presence or magnitude of anaesthetic-induced cognitive impairment depends on the agents used, determination of those differences would enable researchers to make informed choices.

Furthermore, there are some conditions that are set by the objectives of the particular experiment, such as the sex or developmental life stage of the subjects. Although researchers cannot vary these conditions to reduce the confounding effects of anaesthesia, knowledge of how the expression of anaesthetic-induced cognitive impairment is affected by these conditions could facilitate interpretation and comparison of experimental results.

Aim 3: to better understand the neuronal mechanisms underpinning anaesthetic-induced cognitive impairment.

A variety of anaesthetic agents have been shown to cause apoptotic neuronal cell death when administered to neonatal animals (for an expert working group report on developmental anaesthetic neurotoxicity see (Jevtovic-Todorovic et al., 2013)). It appears, however, that although neuronal apoptosis may be *sufficient* to cause anaesthetic-induced cognitive impairment it may not be *necessary* in all circumstances. Anaesthetic exposure is also known to induce a variety of other effects such as alterations in neurogenesis, up regulation of inflammatory markers and structural changes in the surviving (non-apoptotic) neurones. It is thought that vulnerability to anaesthetic-induced neuronal apoptosis is related to the peak of synaptogenesis that occurs during development (Jevtovic-Todorovic et al., 2013), and that this potential mechanism may not be relevant to young adult animals.

Therefore, understanding the changes that are induced in surviving neurons, such as alterations in dendritic ultrastructure, is important if the ways in which anaesthesia may act as a confound for neuroscience experiments is to be elucidated.

Mechanisms of general anaesthesia

What is anaesthesia?

First introduced by Sir Oliver Wendell Holmes, a Professor of Anatomy and Physiology, the term anaesthesia has been in common use since 1846 (Horine, 1946), and refers to the induction of a reversible state of insensibility to noxious stimuli. *General* anaesthesia - as opposed to sedation, local or regional anaesthesia - is said to involve a loss of consciousness, and is what is referred to throughout this thesis when the term anaesthesia is used. The endpoints of anaesthesia have been variously described (Davidson, 2006). In the 1950s Rees & Gray coined the term 'anaesthetic triad' referring to the hypnosis, analgesia and muscle relaxation induced to a greater or lesser degree by most anaesthetic agents (Rees and Gray, 1950). Since then a number of other components have been suggested as anaesthetic endpoints e.g., lack of movement, reduction of autonomic reflexes and amnesia; and alternative ways of describing anaesthesia, such as from the biochemical or neurophysiologic action of anaesthetic agents, have been proposed (Davidson, 2006). Discussion around the definition of anaesthesia will continue for many years to come, not least because the state of anaesthesia is not unitary or linear, involving the loss and regain of different physiological and psychological functions at different points in the process, depending on factors such as agent, species and pathological condition.

Apart from the use of hypothermia (predominantly used for the newborn), anaesthesia is induced by administration of pharmaceutical agents, either alone or in combination. Anaesthetic drugs possess a wide range of chemical structures, from elemental (e.g.,

xenon or argon) to complex compounds such as halogenated ethers (e.g., isoflurane or sevoflurane), barbiturates (e.g., pentobarbitone) and phenols (e.g., propofol); and occur in a variety of states (gases, volatile and non-volatile liquids, or solid powders) at room temperature and atmospheric pressure (Kopp Lugli et al., 2009). Unsurprisingly, for such a diverse group of drugs, anaesthetic agents may be administered by many routes, including subcutaneous, intramuscular, intraperitoneal, intravenous, intrathecal, and inhalational, as well as immersion (for fish and amphibians). Despite all of this variation, as a group of drugs they share a common and fairly unique quality - they produce very similar endpoints (even if in a different sequence), reliably in almost all subjects, regardless of species, stage of development, sex and physiological or pathological state. Providing that an appropriate quantity of anaesthetic agent is administered, it is likely that insensibility to noxious stimuli will be produced in a range of subjects as diverse as mammals (e.g., primates, carnivores, rodents, whales and hoofed mammals), amphibians, reptiles, fish, birds and invertebrates.

Loss of consciousness is a key desired end point of anaesthesia (regardless of the species being anaesthetised), the definition and measurement of which are highly problematic. In humans a commonly used surrogate is ‘loss of response to verbal command’. Notably there is a strong correlation between the blood concentration of an anaesthetic agent that causes loss of response to verbal command in humans and that which causes ‘loss of righting reflex’ (loss of ability to right itself when placed in lateral recumbency) in animals (Franks, 2008) (Fig 1.1b). Therefore, loss of righting reflex is a commonly used surrogate for anaesthetic induced loss of consciousness in animal species. Each anaesthetic agent brings about the switch from consciousness to unconsciousness (or at least from responsiveness to

unresponsiveness) over a very narrow and reproducible dose range (Franks, 2008) (Fig 1.1a). This dose range is so narrow that some have referred to the state of anaesthesia as a binary phenomenon, where the depth of anaesthesia is simply either appropriate or inappropriate (Davidson, 2006). Such an approach is probably too simplistic, as the various cognitive and non-cognitive functions appear to be suppressed in a different order depending on a range of variables other than dose, but the fact remains that there are not many other classes of drug that provide that same level of predictability.

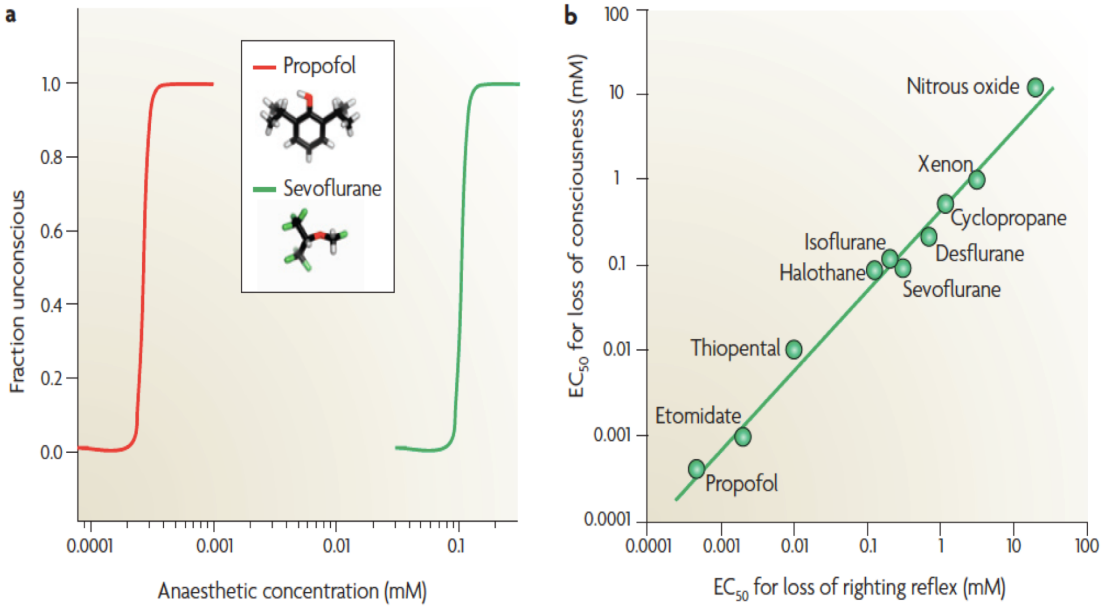

Figure 1.1a and b: Reproduced with permission from (Franks, 2008). Loss of consciousness in humans occurs over a very narrow range of anaesthetic concentrations and correlates with loss of the righting reflex in rodents. a: The concentration-response curves for anaesthetic-induced loss of consciousness are extremely steep. Many factors, including genetic variation, pharmacokinetics and age, tend to broaden population concentration-response curves; when age, in particular, is taken into account, the switch between the conscious and unconscious states can be seen to occur over a very narrow range of concentrations for a given anaesthetic. These data are from two studies in which patients were grouped according to age: the data for sevoflurane (Katoh et al., 2000) are from patients aged 18-39 years; those for propofol (Smith et al., 1994) are from patients aged 17-49 years. b: The correlation between the anaesthetic concentrations that are needed to cause a loss of consciousness in humans (failure to respond to a verbal command) and those that are needed to cause a loss of the righting reflex in rats and mice. The concentrations that are given are millimolar concentrations in physiological saline at 37C and, for the intravenous anaesthetics, they take protein binding into account.

Anaesthetic-induced loss of consciousness

The scientific field of 'consciousness' is vast, and outside the scope of this thesis introduction. However, there are several related, and not necessarily mutually exclusive, theories of anaesthetic-induced loss of consciousness that are worth distilling.

The global effects of most anaesthetic agents on the brain include depression of cerebral blood flow and glucose metabolism, as well as an overall slowing and increased amplitude of electroencephalographic (EEG) activity (Mashour et al., 2005). However, *in vivo* electrophysiological recording in animals has suggested the thalamus as a specific anatomical target for disruption of sensory information by anaesthetic agents (Angel, 1993). Functional brain imaging in humans support the relatively selective deactivation of the thalamus (and midbrain reticular formation) that occurs at, or during, anaesthetic-induced loss of consciousness (Franks, 2008, Fiset et al., 1999). This evidence has led to the theory of a "thalamocortical switch" (the induction of hyperpolarisation of thalamocortical neurons and a discrete transition from tonic to burst firing), as the neurophysiologic basis of anaesthetic-induced loss of consciousness (Alkire et al., 2000). However, it seems unlikely that thalamocortical networks remain 'switched off' for the duration of anaesthesia. A variety of experimental modalities have provided evidence from both humans and animals that the anaesthetised brain continues to process auditory and visual stimuli, which are probably processed through the thalamus (Hudetz, 2006). In addition, ketamine (a drug which tends to be the exception to most rules) does not suppress sensory evoked potentials or EEG, and so would appear to enable some cortical

processing to continue, despite inducing a state of non-responsiveness at high doses (Hudetz, 2006).

Cognitive binding is a term used to describe the ability of the brain to amalgamate sensory information into a unified perception, and is thought to be a crucial aspect of consciousness (Crick and Koch, 2003). "Cognitive *Un*binding" has been proposed as a potential mechanism of anaesthetic-induced loss of consciousness (Mashour, 2013). This theory is supported by the evidence that a number of anaesthetic agents, possessing different receptor targets, induce a breakdown (*un*binding) of fronto-parietal communication (Lee et al., 2009, Ku et al., 2011).

Top down attentional processing from the prefrontal cortex to more posterior cortical and subcortical areas is important for appropriate selection and enhancement of relevant sensory input (Sarter et al., 2001) (a type of information integration), and is thought to potentiate conscious experience (Changeux, 2012). A theory highly related to Cognitive Unbinding is based on the disruption of information integration. Tononi's theory of consciousness, the "information integration theory" proposes that consciousness corresponds to the capacity of a system to integrate information (Tononi, 2004). Increasing evidence supports the view that anaesthetic agents induce wide spread disruption of cerebral network functional integration (Heinke and Koelsch, 2005). The extent to which this disruption is specific to particular networks or neuronal transmission states is still unknown, but the possibility that anaesthetic-induced loss of consciousness could involve non-specific induction of an imbalance between excitatory and inhibitory neuronal signals, has not yet been excluded.

A non-directional disruption of neuronal function could provide an explanation for one of the many remaining puzzles relating to the molecular mechanisms of anaesthetic drugs. Anaesthetic agents that bind to GABAa [gamma-aminobutyric acid type a] receptors produce sedation and loss of consciousness, regardless of the developmental stage (of the animal) during which the drugs are administered. This is despite the fact that activation of GABAa receptors during development depolarises neurons and hence facilitates excitation, as opposed to the inhibition (via hyperpolarisation) that is facilitated during adulthood (Ben-Ari, 2002). This phenomenon could be explained if there was a critical balance between excitatory and inhibitory signals within brain networks that is susceptible to disruption (in either direction) by anaesthetic agents. Indeed, anaesthetic agents can produce a state of non-responsiveness to external stimuli in species that do not possess complex neocortical architecture, suggesting that the state of general anaesthesia may not be dependent on the presence of particular anatomic areas. The investigation of the mechanisms of anaesthesia through the neuro-anatomic parcellation of their effects in humans, may therefore be of limited translational value to some other animal species.

The site(s) of anaesthetic drug action

In the early 1900s Meyer and Overton independently observed a strong correlation between the potency of an anaesthetic agent and its lipid solubility, with the most lipophilic drugs being the most potent. This almost linear correlation holds true for agents with lipid solubilities over 5 orders of magnitude and is known to this day as the Meyer-Overton correlation (Kopp Lugli et al., 2009) (Fig 1.2). This discovery

suggested a common mechanism for anaesthetic agents and was developed into theories focussed on the structural disruption of neuronal membrane lipid bilayers by anaesthetic agents (Ueda et al., 1986, Trudell, 1977, Mori et al., 1984).

However, problems exist with the Meyer-Overton hypothesis. One of these is that anaesthetic agents cause only slight changes in the structure of lipids, which can be reproduced by increases in temperature, that not only do not cause alterations in mentation but actually increase the anaesthetic dose needed to bring about a loss of consciousness (Franks and Lieb, 1982). In addition, many injectable (as opposed to inhalational) anaesthetic agents do not fit the significant relationship between lipid solubility and potency.

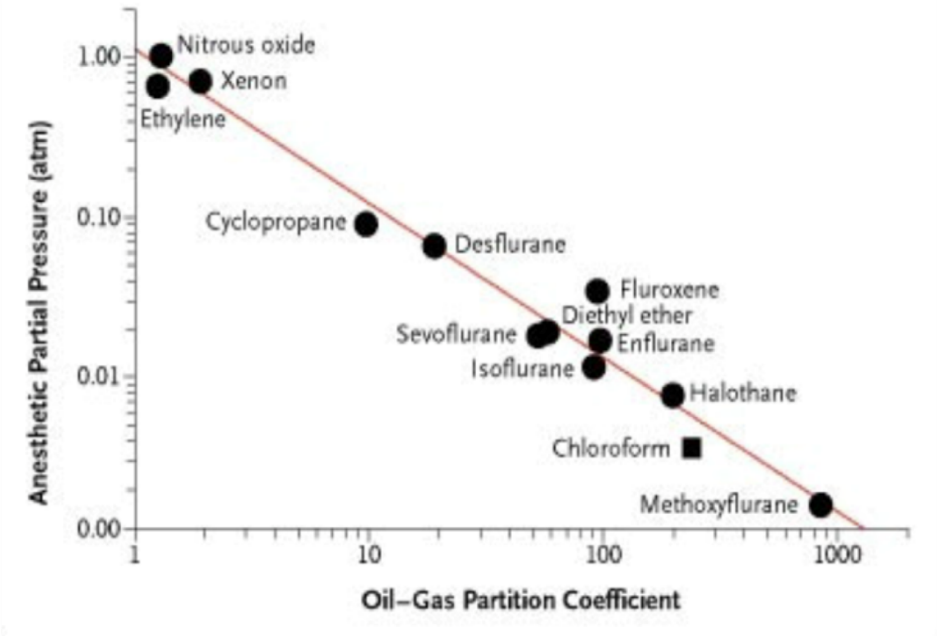

Figure 1.2: Reproduced with permission from (Campagna et al., 2003). A representation of the Meyer-Overton correlation between anaesthetic potency, with respect to a single end point, and the hydrophobicity of the anaesthetics shown. The partial pressures of inhaled anaesthetic, required to prevent movement in response to a surgical incision in humans, is plotted against the olive oil-gas partition coefficient. Chloroform data are based on studies in animals (Campagna et al., 2003). Although it works for most anaesthetics the Meyer-Overton rule fails to explain the lack of anaesthetic potency of some related hydrophobic compounds (not depicted in the figure).

For the above reasons other models have been explored. Computer-based molecular modelling has been used to investigate the physiochemical properties of anaesthetic agents and their relationship to potency (Sewell and Halsey, 1998, Abraham et al., 2008). One of these techniques, Comparative Molecular Field Analysis has been used to determine the relationship between the spatial distribution of molecular shape and electrostatic charge of anaesthetic agents, and their associated *in vivo* potency. Models for structurally diverse inhalational and injectable agents demonstrate a high degree of predictive power (Sear, 2009) and provide a potential common molecular basis for mechanistic activity.

An alternative approach to the study of anaesthetic mechanisms is to focus on their binding to specific protein targets (for review see (Franks, 2008)). This form of 'bottom up' investigation has been facilitated by the establishment and increased use of genetically altered mice, as an experimental model. There are currently three types of receptor that have been identified as key molecular targets with a role in anaesthetic- induced loss of consciousness (GABAa and NMDA [N-methyl-D-aspartate] receptors plus 2PK [two-pore-domain potassium] channels) (Fig 1.3). Almost all anaesthetic agents have been found to potentiate GABA-induced chloride currents, and directly activate GABAa receptors at higher concentrations. Most inhalational, and some injectable, anaesthetic agents inhibit NMDA receptors, which mediate the slow components of excitatory synaptic transmission. Activation of 2PK channels appears to account for at least some of the effects of volatile anaesthetic agents.

Other targets implicated in anaesthetic- induced loss of consciousness include other ligand-gated ionotropic neurotransmitter receptors such as acetylcholine, serotonin,

AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors (Alkire et al., 2008) (Fig 1.3), as well as cyclic-nucleotide-gated and sodium channels (Franks, 2008), all of which are differentially sensitive depending on the agent. Receptor effects vary from minor or major potentiation / inhibition to biphasic effects and effect the function of a range of cellular organelles, as well as neuronal gene and protein expression. Anaesthetic agents also bind to a range of receptors that are involved in the other anaesthetic endpoints (such as suppression of nociception or autonomic reflexes), and may also have a variety of 'off target' effects that have yet to be discovered.

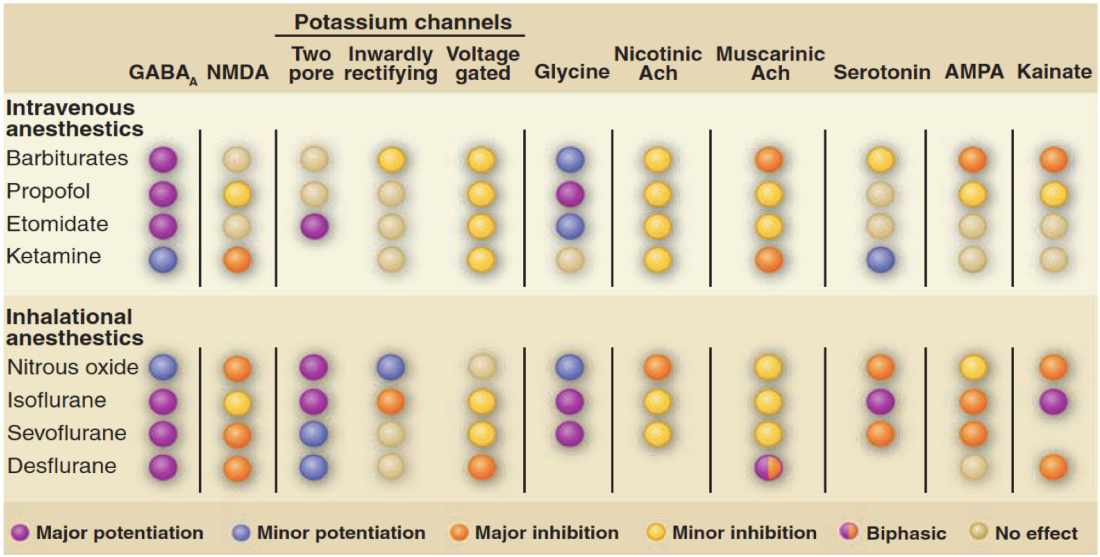

Figure 1.3: Reproduced with permission from (Alkire et al., 2008). Ionic mechanisms and targets of clinical anaesthetics. Abbreviations: Ach, acetylcholine; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABAa, gamma-aminobutyric acid type a; NMDA, N-methyl-D-aspartate.

The gross anatomical targets for anaesthetic agents also vary depending on the end point. Goats have a unique cerebral circulation (lacking internal jugular veins and direct vertebral artery contributions to the brain), making it possible to isolate cerebral vasculature in the living animal. This feature has been capitalised by Antognini and colleagues, who established a model that enabled isolated exposure of the brain and

head to doses of anaesthetic agent (Antognini and Schwartz, 1993). Use of this model has provided compelling evidence that anaesthetic effects on the spinal cord account for a significant degree of the loss of movement responses associated with induction of anaesthesia (by suppressing nociceptive stimuli before they reach the brain), *and* contribute to (but do not account for) the suppression of arousal (Antognini et al., 2005, Mashour et al., 2005). Therefore, the brain is not the only gross anatomical target but rather anaesthetic drugs target the central nervous system as a whole. Of course systemic administration of anaesthetic drugs necessarily results in effects (many unwanted) throughout the entire body at multiple levels (from the molecular, organelle and cellular, to brain networks and behaviour). Our understanding of the effects at each level continues to expand rapidly but there is still much to learn about how these effects integrate to produce the state we refer to as general anaesthesia.

General anaesthesia and animal research

Laboratory animal anaesthesia as a discipline

General anaesthesia has been used to facilitate human surgical procedures since at least the mid-1800s; although laboratory animal anaesthesia may have a history that predates this (for a brief overview of the inception of modern day anaesthetic practice please refer to Appendix 3). Researchers and government officials increasingly recognise how important good quality laboratory animal anaesthetic practice is for both animal welfare and scientific validity. And in modern times laboratory animal anaesthesia is a fast-growing discipline, now featuring as specialised book chapters within authoritative veterinary anaesthesia (Tranquilli W. J.' Thurmon J. C., 2013) and biomedical research (Murphy et al., 2012) text books.

Of the 4 million animals used in biomedical research each year in the UK alone, one quarter of them undergo general anaesthesia as part of animal model development (Statistics, 2013). The ideal anaesthetic regimen eliminates pain and distress without harm to the animal and has no effect, either direct or indirect, on the scientific data produced. Unfortunately however, all anaesthetic agents depress cardiovascular and respiratory function to a greater or lesser extent, and interfere with other physiological mechanisms such as thermoregulation and acid-base balance, inflammatory and immune system processes. Best practice in laboratory animal anaesthesia continually evolves as new techniques and drug combinations become available, in order to reduce these unwanted effects. This process of *refinement* of anaesthetic regimens can greatly

enhance animal welfare by reducing the physiological impairment induced by anaesthesia and the consequences thereof.

In addition to the enhancement of animal welfare through the refinement of anaesthetic regimens, in order for maximal benefit of the use of animals in biomedical research to be realised, scientific validity must be maintained. It is therefore essential to understand any confounding effects that anaesthesia, and changes in anaesthetic protocols, may have on the biological processes involved. Off target (unintended) effects of anaesthesia may alter physiological processes, either directly or indirectly, at many different levels and therefore potentially interfere with a wide range of scientific aims and objectives. During laboratory animal anaesthesia (as appose to clinical anaesthesia), the need to minimise any confounding effects of anaesthesia on the scientific output adds a layer of complexity to the choice of techniques and regimens. Best practice in laboratory animal anaesthesia is therefore dependent on investigation of anaesthesia as a confound for *in-vivo* animal experiments.

Anaesthesia as a confound for neuroscience animal experiments

There are a number of ways in which anaesthesia, or rather the type of anaesthetic agent used, may interfere with experimental outcomes. One area where this is illustrated is the collection of blood oxygen level dependent (BOLD) response data during functional magnetic resonance imaging (fMRI). Historically alpha-chloralose was a popular anaesthetic for rodents undergoing fMRI procedures because of its ability to induce non-responsiveness without depressing the BOLD signal to the same extent as volatile anaesthetic agents. However, the effect of alpha-chloralose on the

BOLD signal has been found to vary during extended periods of anaesthesia, which could complicate data interpretation (Austin et al., 2005). Austin and colleagues studied the amplitude of the BOLD response, to direct cortical stimulation, during a single episode of anaesthesia. For the first part of the anaesthetic episode halothane (a volatile agent) was administered at a variety of doses, animals were then transferred to alpha-chloralose anaesthesia without undergoing recovery from anaesthesia. The authors observed that halothane anaesthesia was associated with a stable BOLD response to stimulation, regardless of the dose administered. However, alpha-chloralose anaesthesia was associated with a slow increase in BOLD signal response (spatial extent and peak level) over a 6-hour period.

Alpha-chloralose anaesthesia was maintained with intermittent bolus injections of agent, that would presumably have led to a 'see-saw' variation in depth of anaesthesia, rather than a gradual linear change throughout the 6-hour period; and so the variation in BOLD signal response did not appear to be associated with the depth of anaesthesia. Ideally this result would be followed up with a similar experiment that investigates the two agents being given in the opposite order (as transition from one agent to the other could have confounded the results). Nonetheless this is an interesting observation that suggests that the BOLD signal response under anaesthesia may be agent dependent.

Further to such gross effects on the BOLD signal, more subtle, modular, effects can confuse data interpretation. In 2007 Vincent et. al. investigated intrinsic functional architecture in anaesthetised macaques, under different doses of isoflurane. The authors' focus was that particular fronto-parietal functional MRI connections were conserved at different depths of anaesthesia (e.g., between frontal eye field and the

lateral intra-parietal cortex). They therefore concluded that anaesthetic dose was not a critical variable for the fMRI experiment. However, not noted by the authors, the data also demonstrated that some other connections, e.g., between dorsolateral prefrontal cortex and lateral intra-parietal cortex were abolished with a higher (but not lower) dose of isoflurane (Vincent et al., 2007). Dose-related differential effects of anaesthesia on functional connectivity have also been demonstrated in humans (Mhuircheartaigh et al., 2010). Given that common practice is to collect fMRI data sets at one particular dose of anaesthesia, if a dose of isoflurane equivalent to the higher dose used by Vincent and colleagues were to be used, and anaesthesia was not considered to be a critical variable, investigators could mistakenly conclude that particular functional connections do not occur in the macaque.

These examples illustrate how anaesthesia may interfere with the extent and magnitude of a desired response whilst the drugs are being administered. However, anaesthesia may also have enduring effects. It is well established that anaesthesia has short term effects on cognition but an increasing body of work has shown that anaesthesia may also have longer term effects on learning and memory beyond the point at which the drugs are excreted from the body (Jevtovic-Todorovic et al., 2013). A lot of resistance was initially encountered to the view that an organism is not returned to its previous state following recovery from general anaesthesia. But as well as having implications for clinical patients and patient selection for surgery, this implicates anaesthesia as a critical variable for the design of behavioural neuroscience experiments. Investigation of these effects then becomes essential, in order to gather the empirical evidence required to enable researchers to make informed decisions about the use of different anaesthetic regimens for *in-vivo* experiments.

The first step to understanding what these enduring effects are, is to understand which psychological processes are effected, as this is essential for phenotypic characterisation. Existing literature examining, and providing evidence for, the enduring effects of anaesthetic exposure during adulthood (as opposed to development), is focussed on a relatively limited array of behavioural tasks. This literature is discussed below, and has been delineated according to the particular behavioural tasks used, in order to lay the building blocks for the phenotypic characterisation of anaesthetic-induced cognitive impairment, and specifically to highlight any gaps in knowledge.

Anaesthetic-induced cognitive impairment in young adult animals

Spatial working memory

Exposure of young adult rodents to commonly used anaesthetic agents is sufficient to produce post-anaesthetic spatial memory impairment (Culley et al., 2004b, Wiklund et al., 2009, Bianchi et al., 2008, Le Freche et al., 2012, Zurek et al., 2012, Callaway et al., 2012b,, Saab et al., 2010). Some of the earliest studies to investigate this looked at the effects of anaesthesia on spatial working memory in rodents (Culley et al., 2004b, Culley et al., 2003a, Crosby et al., 2005). Reproduction of the effect reported in these studies provided an anchor point for the work of this thesis.

Six-month-old rats anaesthetised for two hours with 1.2% isoflurane plus 30% nitrous oxide and 70% oxygen demonstrate spatial working memory impairment, compared to control rats that received 30% oxygen in an identical chamber (Culley et al., 2004b). The task chosen for this study was a win-shift spatial working memory task

on a 12- arm radial arm maze. For the win-shift task, each of the arms of the maze are baited with food rewards at the beginning of each trial but are not replaced within a trial. Thus, rats have to keep track of locations visited within a trial in order to obtain the remaining food rewards efficiently (but note they are not required to remember the spatial location of the food rewards across trials). Win-shift radial arm maze testing began 48 hours after anaesthesia and rats underwent one trial per day for 21 days post- anaesthesia. Previously anaesthetised rats were slower to complete the maze, and made fewer correct choices before the first error was made, than control rats. As the majority of isoflurane is cleared from the brain within minutes of cessation of a 2-hour anaesthetic exposure (Bailey, 1997, Chen et al., 1992) and only trace amounts are present 24 hours later (Saab et al., 2010), it is unlikely that this impairment in performance is due to residual anaesthetic (48 hours after exposure). Swim speed was assessed in separate groups of rats and found to be similar (Culley et al., 2004b), so it is also unlikely that differences in locomotive ability account for the differences in maze performance, although the motor function involved in swimming tasks is different to that involved in dry maze tasks, so that conclusion cannot be definitive.

The win-shift radial arm maze task used in the above experiment involves recognition and discrimination between previously visited and unvisited locations. It capitalises on the innate behaviour of rodents, used during foraging, to preferentially explore unvisited locations. Because the task involves the presentation of multiple familiar stimuli (rats being acclimatised to the arms of the maze prior to testing so that they are familiar) and requires the ability to remember multiple locations within a trial, the psychological process tested has been thought for a long time to be operationally similar to working memory in humans (Olton, 1987). However, it is also possible that

task performance relies on relative familiarity judgements based on the ability to habituate to recently visited locations (for discussion see (Sanderson and Bannerman, 2012)) i.e., short term memory but not working memory *per se*, to the extent that working memory implies the active maintenance of information versus passive recognition of places visited (Castner et al., 2004). In either case, performance relies on the use of extra-maze spatial cues (Olton, 1987, Gallagher et al., 1983) and is dependent on hippocampal function (Winocur, 1982).

Depending on the RAM testing paradigm, other brain areas such as the prefrontal cortex and the striatum, may be engaged for efficient task performance (Floresco et al., 1997), although lesions of the prefrontal cortex do not always affect task performance (discussed in (Sanderson and Bannerman, 2012)). It has been suggested that choice accuracy on the radial arm maze is crucially dependent on the cholinergic neurotransmitter system (Levin et al., 1989, Walsh et al., 1984) and may also be modified by other neurotransmitter systems (Taghzouti et al., 1986, Decker and Gallagher, 1987, McGurk et al., 1988). However, rats receiving highly selective lesions of cholinergic neurons in all major areas of the basal forebrain, demonstrate no impairment in RAM working memory (Vuckovich et al., 2004), supporting the view that working memory performance on the RAM is not dependent on the cholinergic neurotransmitter system.

The study conducted by Culley and colleagues (that established an anaesthetic-induced impairment in win-shift spatial memory, in young adult rats) found that 18-month-old rats, tested within the same experimental paradigm as young adult rats, also demonstrated post-anaesthetic cognitive impairment (Culley et al., 2004b). A later study by the same group examined whether the impairment in win-shift spatial

working memory, in aged rats, was persistent and dependent on the use of nitrous oxide. They found that aged rats that received either isoflurane or isoflurane plus nitrous oxide were slower to complete the radial arm maze and made fewer correct choices before the first error occurred (compared to controls), even when testing began 2 weeks after they were exposed to anaesthesia (Culley et al., 2004a). This persistence of effect was *not* found when young adult rats began testing 2 weeks after anaesthesia, suggesting that the duration of effects of anaesthesia on post-anaesthetic cognitive performance are age dependent (Crosby et al., 2005).

Using the same experimental design as the Culley studies (but with 14 days rather than 21 days of testing), Crosby and colleagues (Crosby et al., 2005) found that there were no group differences, in the time to complete the maze or the number of correct choices before the first error occurred, for young adult rats. However, when they looked at the total number of errors made within each trial they found an interaction between anaesthesia group and testing day that reflected a greater reduction in errors across training for the group anaesthetised with isoflurane plus nitrous oxide. The authors concluded, that previous anaesthesia with isoflurane and nitrous oxide actually *facilitated*, not impaired, maze performance when testing began 2 weeks after anaesthesia.

This conclusion appears to contradict that of Culley and colleagues, however, an element of caution should probably be used when considering this conclusion, as the effect appears to be small and the control group don't demonstrate an improvement across testing days. The lack of improvement across testing days would not necessarily be a concern for a win-shift task because the behaviour is spontaneous and animals are not necessarily required to learn information between trials.

However, *exactly* the same experimental apparatus and methods (personal communication (Culley, 2008)) have been used for other experiments, where young adult controls of the same strain demonstrate a dramatic improvement in performance across testing days (e.g., (Culley et al., 2004b)). Notably (in Crosby 2005), the anaesthesia treated rats were performing numerically worse than the control rats at the start of testing, which makes interpretation of group differences difficult. The possibility that the controls did not perform as expected during this particular experiment cannot be entirely ruled out therefore. Indeed, studies conducted by other groups suggest that young adult rodents exposed to anaesthesia do demonstrate post-anaesthetic impairment (not a facilitation) in spatial memory, even when testing occurs up to two weeks after anaesthetic exposure (Lin and Zuo, 2011, Le Freche et al., 2012), see below for details). So even though the literature for anaesthetic-induced impairment of radial arm maze performance is not straightforward, the result by Culley and colleagues warrants more investigation.

Spatial reference memory

Reference memory tasks test the ability of subjects to remember specific spatial locations *across* trials, and associate those locations with outcomes (e.g., the presence of food rewards). This process is not tested during the win-shift working memory radial arm maze task. Young adult female mice anaesthetised for only one hour per month for four months, with either 1.5% or 2.5% sevoflurane demonstrated a decreased time spent in the target quadrant (compared to controls) during a MWM probe trial, when testing began 1 week after anaesthesia (Le Freche et al., 2012). These results support the view that anaesthesia induces spatial reference memory

impairment, in addition to the spatial 'working' memory impairment discussed above.

The MWM consists of a circular pool of opacified water, containing a fixed location, escape platform, hidden beneath the surface of the water. During spatial acquisition, the rodent is placed into the pool (at a random location) and must swim around until the escape platform is found. The platform remains in the same location across trials. As the rodent learns the location of the platform over repeated trials the path taken to reach the platform becomes more direct and the path length and time taken become shorter. Once the platform location has been learned, probe trials can be carried out, where the platform is removed and the time that the rodent spends looking for the platform in the correct quadrant of the pool is determined - if the rodent remembers where the platform was it should spend most of its time in that quadrant - for detailed methods refer to (Vorhees and Williams, 2006). The MWM can also be used to assess working memory by way of a different testing paradigm, however this is less commonly undertaken.

In the study by Freche and colleagues (Le Freche et al., 2012), the performance of anaesthetised mice was not different from controls during the acquisition phase of the task but anaesthetised mice *did* spend less time in the target quadrant than controls, during the probe trial 72 hours after the end of the acquisition phase, suggesting that their memory of the platform location decayed to a greater extent than controls (i.e., memory retention was impaired). In a separate study, Callaway and collegues did not find that exposure of young adult rats to sevoflurane (Callaway et al., 2012a) or desflurane (Callaway et al., 2015) was associated with spatial memory impairment (either acquisition or retention) in the MWM, suggesting that the effect

of anaesthesia may be dependent on the agent used, species exposed, or the specific laboratory conditions under which the testing is performed. However, the same group did find that exposure of young adult rats to isoflurane (a different anaesthetic agent) impaired memory retention (but not acquisition) in the MWM (Callaway et al., 2012b), a result that supports that of Freche and colleagues, despite use of a different agent and species. These results do not however rule out the possibility that variation of results within the field could be due to laboratory dependent factors, such as animal housing and husbandry conditions, the salience of spatial cues, or the construction of apparatus.

Persistent post-anaesthetic impairments in spatial reference memory have been suggested with the use of a different reference memory task, carried out on the Barnes maze. Young adult rats anaesthetised for 2 hours with 1.2% isoflurane and 100% oxygen demonstrated an increased time to locate the escape box on a Barnes maze (compared to control rats that received 100% oxygen) when testing began two weeks after anaesthesia (Lin and Zuo, 2011). The Barnes maze (Barnes, 1979) consists of a flat circular tray with a number of holes around its perimeter, one of which (the target hole) leads to a dark escape box. Rats have an innate preference for dark enclosed spaces and so will learn the location of the escape box, when allowed to explore the tray over repeated trials. Visual navigation cues can be either extra maze or proximal cues (placed on the maze and fixed in relation to the escape box) (Harrison et al., 2006), and when extra maze cues are used, the construct of the Barnes maze task is similar to the MWM task described above.

Although the Barnes maze task does involve the use of mild anxiogenic motivating factors such as relative light levels, the results of Lin et. al., suggest that spatial

reference memory impairments occur in rodents in the absence of stronger motivating factors such as food restriction (the radial arm maze) or escape from deep water (MWM). It also suggests that they persist for at least 2 weeks after anaesthetic exposure (a period of time that is of relevance to the design of behavioural neuroscience experiments - where an interval of up to two weeks is commonly used between the surgical and behavioural elements of an experiment). Yet the results of Lin & Zuo are again complicated by the unusual performance of the control group. Short and long-term memory were assessed by testing the ability of subjects to find the escape box, 1 and 8 days after training. Although not statistically compared, control rats have numerically better performance in long-term memory compared to short term memory, an effect that appears to be driving the finding that anaesthetised rats have impaired long-term memory performance (as anaesthetised rats have almost identical numerical performance for both short-term (where no effect of anaesthesia was found) and long-term memory performance (Lin and Zuo, 2011). This study highlights that caution is required when interpreting results from the relatively low number of publications within the field.

The MWM (and Barnes maze) tasks are similar to the previously described win-shift (spatial *working* memory) RAM task in so far that they test allocentric place learning i.e., the rodent utilises extra maze cues to facilitate spatial navigation; a process that is dependent on function of the hippocampus (O'Keefe and Nadel, 1978, Morris et al., 1982). However, importantly they differ in a number of ways. The reference memory component of the MWM (and Barnes maze) tasks described requires subjects to use a psychological process that is different to the 'win-shift' strategy described for the RAM task. Successful performance of these tasks requires learning that a spatial location is associated with a specific outcome (either escape box or

platform) *across* trials (a process that is dependent on cholinergic and glutamatergic neurotransmitter systems (McNamara and Skelton, 1993). Because the location of the box or platform does not change, rodents use a 'win-stay' strategy to do this, where they return to the previously successful spatial location upon successive trials. Interestingly though, differences in the psychological processes involved can occur *within* a memory domain and not just *between* memory domains. The RAM can be used to assess spatial reference memory (as well as spatial working memory), however it is possible that the psychological processes involved in spatial reference memory in the RAM may be different to those in the MWM (Bannerman et al., 2014), highlighting the potential complexity of the psychological processes involved and how they may differ, not only according to the type of memory being assessed but also the apparatus and method being used to assess them.

One point of note from collective consideration of the studies presented above is that they investigated the use of volatile and not injectable anaesthetic agents. Is it possible therefore that only volatile agents are problematic? In support of that view are a number of publications by Antunes and colleagues, who have investigated the effects of administration of ketamine, midazolam or combinations of ketamine with either midazolam or medetomidine (all of which are non-volatile, injectable agents) on the spatial reference memory in rodents, using either the radial arm maze or simpler 'T' or 'Y' maze tasks (Ribeiro et al., 2013, Valentim et al., 2013b, Valentim et al., 2013a). No spatial memory deficits were found in any of the studies, suggesting that non-volatile anaesthetic drugs are not associated with post-anaesthetic cognitive impairment. However, some apparatus and method details from those studies were limited, and positive controls were not included. One notable difference was that the radial arm maze used for those experiments contained 30cm high non-transparent arm

walls (personal communication A Valentim 2009), that probably impaired the ability of subjects to utilise extra-maze visuo-spatial cues. In addition, research from the field of developmental anaesthetic neurotoxicity (a much larger field) suggests that injectable anaesthetic agents *can* induce post-anaesthetic cognitive impairment when administered during development (Jevtovic-Todorovic et al., 2003b), and so it may be unwise to disregard the potential involvement of injectable agents in anaesthetic-induced cognitive impairment.

Fear conditioning

It is not just the performance of navigation tasks that is impaired by exposure to volatile anaesthesia. Fear conditioning paradigms are not used within this thesis, however impairments in these tasks provides evidence for the role of the hippocampus in anaesthetic-induced cognitive impairment. After examining the effect of anaesthesia on spatial reference memory using the Barnes maze task, Lin & Zuo (Lin and Zuo, 2011) used the same groups of rats to assess fear conditioning. Rats were placed individually into a conditioning chamber and allowed to explore for 30 seconds before pairing was repeated twice more before returning the rats to their home cage. Twenty-four hours later, contextual and auditory-cue fear conditioning were tested by returning rats to either the same conditioning chamber in the absence of tone and shock (contextual), or a novel conditioning chamber where the tone (auditory-cue) was presented in the absence of shock, and then measuring the amount of freezing displayed by the animal. Rats have an innate freezing response to signals associated with danger and so will demonstrate increased freezing if they have learned, and remember, that the context and/or the auditory cue are associated with foot shock (Phillips and LeDoux, 1992). The order of contextual and auditory-cue fear

conditioning was counterbalanced across anaesthesia groups. Anaesthetised rats spent less time freezing than control rats during the contextual test and *not* during the auditory-cue fear conditioning test (Lin and Zuo, 2011), when conditioning was carried out 27 days after exposure to anaesthesia.

Fear conditioning is a type of Pavlovian conditioning, where an animal exhibits a conditioned response (freezing) because association is made (learned and remembered) between a conditioned stimulus (either context or auditory cue) and an unconditioned stimulus (foot shock). Most obviously it differs from the maze tasks described above because it does not contain a navigation component. The amygdala plays an important role in the process of fear conditioning regardless of whether the conditioned stimulus is the context or the cue, but the hippocampus is classically thought only to be involved in contextual fear conditioning (Phillips and LeDoux, 1992), demonstrating that the neurobiological substrates involved in these two paradigms may be subtly different.

Isoflurane-induced impairments in contextual (but not auditory-cue) fear conditioning have also been found in mice, when conditioning occurs 24 hours (rather than 27 days as in (Lin and Zuo, 2011)) after anaesthetic exposure. Four-month-old male or female mice, previously exposed to 1.3% isoflurane in 30% oxygen, demonstrate a lower degree of freezing during contextual fear conditioning than control mice exposed to 30% oxygen (Saab et al., 2010). Impairment occurred only in mice that were tested 1 hour and not 48 hours after conditioning (conditioning in both cases was carried out 24 hours after anaesthetic exposure).

Impairment in both contextual *and* auditory-cue fear conditioning was found in separate groups of mice, when conditioning occurred 1 hour after exposure to anaesthesia, regardless of whether the interval between conditioning and testing was 1 hour or 48 hours. However, with conditioning being so close to the cessation of anaesthesia, this part of the study may not be investigating the long-term effects of anaesthetic exposure but rather the direct, acute, effects of exposure. In separate control experiments the authors demonstrate that impairment 1 hour after anaesthetic exposure is not due to alterations in levels of anxiety (as measured using the elevated plus maze), locomotor ability (as measured using an elevated beam task) or nociception (as measured by the tail flick test). They also show that isoflurane levels in brain tissue 1 hour after anaesthetic exposure are below 0.05%, which certainly wouldn't be enough to noticeably sedate subjects. However, a direct effect cannot entirely be ruled out given that the dose of anaesthetic required to induce amnesia may be less than that required to induce non-amnestic end points (Dwyer et al., 1992). Residual isoflurane levels in brain tissue also raise the possibility that the process of retrieval may be impaired rather than those of learning or memory, i.e., a 'state dependent memory' could be present (where memory retrieval is most efficient when the subject is in the same state of consciousness as they were when the memory was formed) (Rosman et al., 1992). In other words, the formation of memory could have occurred when the animal was under the influence of anaesthesia, and would therefore need to be in that same physiological state in order to remember the event (shock-stimulus pairing).

If the results from mice tested 24 hours after anaesthetic exposure (and not 1 hour after anaesthetic exposure) are considered in conjunction with those from Lin & Zuo (above) in rats, they suggest that isoflurane causes post-anaesthetic impairment in fear

conditioning, when testing occurs up to 24 hours but not 48 hours after conditioning and only when a hippocampal dependent paradigm is used. In contrast to these results another study found that rats exposed to 1.8% isoflurane for 2 hours did not demonstrate impairment in contextual fear conditioning when conditioning occurred 24 hours after anaesthetic exposure (Uchimoto et al., 2014). However, rats did demonstrate impairment when conditioning occurred 7 days after anaesthetic exposure, suggesting a progressive impairment. Direct comparison with the studies outlined above is problematic though, as Uchimoto and colleagues used an Inhibitory Avoidance task which measures a different behavioural response to the standard fear conditioning paradigm. The Inhibitory Avoidance task measures the latency for rats to re-enter a dark box (from a connected light box) in which they previously received a context-shock pairing. This behavioural response potentially involves an array of processes including freezing, inhibition, motivation and anxiety. It should also be noted that in the study by Uchimoto and colleagues a shock of 0.8mA lasting 2 seconds was used, which is a relatively high current shock delivered over a much more prolonged period than the other studies. This methodological difference could also potentially affect the behavioural response measured, for instance by inducing alterations in nociception.

Object recognition

Object recognition tasks are not used within this thesis, however impairments in these tasks suggest that the psychological process of novelty preference may be impaired by anaesthesia. An alternative task used to assess novelty preference in rodents is the spontaneous spatial novelty preference task (Sanderson et al., 2007). This task is used

in Chapter 3 here, in order to further investigate the anaesthesia-induced spatial memory deficits reported in existing literature.

Exposure of young adult mice to either 1.3% isoflurane or 2.3% sevoflurane impairs object recognition when testing occurs 24 hours (and not 72 hours) after anaesthesia (Zurek et al., 2012). For behavioural testing, mice were placed individually in a testing chamber that consisted of opaque walls and contained two identical objects (e.g., toy cars), and were allowed to explore both objects for up to 10 minutes before being returned to the home cage. After either one minute or one hour (i.e., a variable retention delay) each mouse was placed back into the testing chamber but this time there was one familiar object (e.g., toy car) and one unfamiliar object (e.g., configuration of toy building blocks). The time taken exploring both objects was recorded and recognition of the object was deemed to have occurred if time spent exploring the novel object was longer than the time spent exploring the familiar object. This type of spontaneous object recognition task relies on an innate preference of rodents to explore novel rather than familiar objects (Ennaceur and Delacour, 1988). Unlike the previously described tasks it does not require an explicit reinforcement or external motivational factor and so could potentially provide a testing paradigm that is less confounded by any potential difference between groups in such factors.

Early evidence supported a necessary role for the hippocampus in object recognition (Squire, 1992). However, more recent studies have shown that the hippocampus is not always essential for task performance (Winters et al., 2004, Forwood et al., 2005) and indicate that the perirhinal cortex is a critical region for object recognition memory (Winters et al., 2008), with muscarinic cholinergic and glutamatergic receptor subtypes being differentially important for the memory stages of encoding

and retrieval (Winters et al., 2008). Because the hippocampus is known to be important for spatial and context dependent memory (O'Keefe and Nadel, 1978, Hirsh, 1974), it could be viewed that it is only engaged in tasks that contain a strong spatial or contextual component. However, while it has been shown that rats with bilateral lesions of the hippocampus are not often impaired at a standard object recognition task, they *are* impaired at a version that tests the subjects' ability to detect the recency of an encountered object (rats normally demonstrate a preference to explore older less familiar objects over more recent familiar objects) (Albasser et al., 2012). This recency version of the task contains minimal spatial or contextual factors (Albasser et al., 2012) and so the results not only support that the hippocampus is necessary under some conditions for object recognition, but that it can be necessary for non-spatial or contextual memory components, such as temporal order.

With regard to the role of the hippocampus in anaesthetic-induced cognitive impairment, in the study by Zurek and colleagues (discussed above) (Zurek et al., 2012) a relatively small testing box, with opaque walls, was used in an attempt to reduce the spatial and contextual components of the task. The anaesthetic-induced impairment demonstrated therefore could suggest that anaesthetic-induced cognitive impairment may not always be hippocampal dependent. This view cannot be conclusive, however, as hippocampal dysfunction could potentially interfere with task performance even if the hippocampus is not necessary under all conditions.

Only mice that were tested following a one hour and not a one minute retention delay were impaired (Zurek et al., 2012). The authors concluded that anaesthesia impairs consolidation of the object recognition memory trace and not encoding. However, it was reported that following the exposure phase one object was removed and replaced

with a novel object (as opposed to replacing both objects with one identical to the familiar object and one novel object), and so the influence of confounding olfactory cues at a time point so close to when the rat encountered the objects, cannot be ruled out. A different group has used longer delays between anaesthetic exposure and object recognition testing (4 weeks and 10 weeks post-anaesthetic exposure), to test rats previously exposed to isoflurane. No impairment was demonstrated (Zhu et al., 2010), supporting the finding by Zurek and colleagues that isoflurane-induced impairment in object recognition persists for less than 72 hours. Zurek and colleagues have since demonstrated that mice exposed to sedative doses of the injectable anaesthetic agent etomidate (but not sedative doses of the injectable anaesthetic agent dexmedetomidine) demonstrate impairment in object recognition 24 *and* 72 hours after anaesthetic exposure (Zurek et al., 2014), suggesting that the duration of anaesthetic-induced impairment may be agent-dependent.

In a separate experiment mice exposed to 2.6% sevoflurane for 2 hours immediately after exploration of two identical objects demonstrated impaired object recognition when testing occurred 24 hours after anaesthetic exposure (Wiklund et al., 2009). These results would seem to suggest that anaesthesia could induce a retrograde memory impairment - impairment in the ability to remember information encountered *before* anaesthetic exposure. However, it is possible that impairment may not be directly related to anaesthesia but rather reflect an inability for anaesthetised mice to benefit from sleep-dependent memory consolidation (Stickgold, 2005) during the 24 hours between exposure to the objects and testing of recognition of the objects.

The field of anaesthetic-induced cognitive impairment in young adult animals contains a relatively low number of publications, regardless of the cognitive domain being

examined. The extent to which cross study comparison therefore contributes to the characterisation of the behavioural phenotype is limited, because differences in laboratory conditions or animal care, for instance, could account for existing inconsistencies. It is therefore difficult to identify domain specific characteristics but deficits in spatial memory do appear to be a feature. Importantly, what is not known is which psychological processes are responsible for the reported impairments in spatial memory. Further characterisation of those impairments would facilitate identification of the underlying neuronal mechanisms.

Exposure to anaesthesia during development

It is widely accepted that the magnitude of the phenotype of anaesthetic-induced cognitive impairment is greater at the extremes of age (Culley et al., 2007, Jevtovic-Todorovic et al., 2013). Data supporting the existence of anaesthetic-induced cognitive impairment in neonatal animals are compelling (Jevtovic-Todorovic et al., 2013, Jevtovic-Todorovic, 2013). In 2003 a landmark publication, that established the field of developmental anaesthetic neurotoxicity, authors investigated the effect of a combination of isoflurane, nitrous oxide and midazolam - a mixture of volatile, gaseous and injectable anaesthetic agents - on spatial memory. A win-shift radial arm maze task was used to assess spatial working memory and a MWM task to assess spatial reference memory, along with a variety of other ‘control’ tasks to assess the effect of anaesthesia on non-cognitive function.

The authors found that rats exposed to a 6-hour duration anaesthetic at postnatal (P) 7, demonstrated both working and reference memory impairment when testing

occurred at much later time points (P28 and P131 for MWM testing and P53 for radial arm maze testing) (Jevtovic-Todorovic et al., 2003b). Within the same set of experiments the authors used activated caspase-3 and silver staining of post-mortem samples to demonstrate widespread apoptotic neurodegeneration, throughout cortical and subcortical areas (a characteristic feature that has been reproduced in many studies since (Jevtovic-Todorovic and Olney, 2008). In a comprehensive body of work Jevtovic-Todorovic and colleagues have gone on to examine the mechanism of apoptotic neuronal cell death and the effect of anaesthetics on other outcome measures such as organelle structure and function, synaptic ultrastructure and the expression of inflammatory markers (e.g., (Jevtovic-Todorovic et al., 2012, Boscolo et al., 2013a, Rizzi et al., 2010, Lunardi et al., 2011).

Although many within the field have concluded that anaesthetic exposure during development leads to cognitive impairment in rodents (Jevtovic-Todorovic et al., 2013), meta-analysis of the literature does not support this (unpublished data from the 'Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies' - (Stewart, in prep)). In any case, in an area of research that is far more prolific than investigation of the effects of anaesthetic exposure during adulthood, it is notable that spatial memory deficits predominate. It is unclear, of course, whether this reflects the robustness of spatial memory deficits compared to deficits in other cognitive domains, or whether this is simply a function of a previously established effect being used to demonstrate 'cognitive impairment', where the focus of investigation is the mechanism thereof. Both of these factors probably play a role. Because the focus of these studies is not the determination of the psychological processes involved, but rather the investigation of cognitive impairment as a binary function (present / not present), spatial tasks provide a

convenient tool, being relatively easy and quick to use. As with effects in adult animals, phenotypic characterisation of effects of anaesthetic exposure during development is important, however, in order to inform the design of mechanistic investigations, prospective human patient studies and animal behavioural neuroscience experiments.

Potential neuronal mechanisms of anaesthetic-induced cognitive impairment

Historically anaesthetic agents have been thought of as neuroprotective, rather than neurotoxic. For example, barbiturates are known to offer protection when present during a focal ischemic insult (Michenfelder and Theye, 1973), a property that is exploited in clinical practice to this day. Other studies have shown improved outcome in animals subjected to a variety of ischemic / traumatic brain and spinal cord insults, when those insults occurred in the presence of a variety of anaesthetics (Miura et al., 1998, Gelb et al. 2002). Investigation of the mechanisms of these neuroprotective properties continues to focus on the suppression of cerebral metabolic demand (Newberg and Michenfelder, 1983, Eberspacher et al., 2006), neuronal necrosis (Warner et al., 1986) and more recently on the processes of preconditioning (where exposure to anaesthesia provides protection against subsequent pathological challenge) (Zheng and Zuo, 2004).

It has more recently been recognised that anaesthetic agents can also have neurotoxic effects. Exposure of rats to N-methyl-D-aspartate antagonists (e.g., ketamine) causes pathomorphological changes in neurons (Jevtovic-Todorovic et al., 1998, Olney et al., 1989) that can be irreversible at high doses (Olney et al., 1991). Neonatal animals appear to be particularly vulnerable, with the evidence for induction of neuronal apoptosis following exposure to ketamine, and a range of other anaesthetic agents, during development being particularly compelling (Jevtovic-Todorovic et al., 2013). Rats exposed to a combination of isoflurane, nitrous oxide and midazolam anaesthesia, at postnatal day 7, demonstrate spatial memory deficits during adulthood that are associated with apoptosis in cortical and subcortical brain areas (Jevtovic-Todorovic

et al., 2003b) (Fig 1.4). At the other end of the life stage scale, the commonly used volatile anaesthetic agent, isoflurane, induces a vicious cycle of neuronal apoptosis and amyloid ß-protein accumulation when administered to elderly mice (Xie et al., 2007). These neurotoxic properties of anaesthetic agents have been associated with cognitive impairment in animal models (Culley et al., 2007, Jevtovic-Todorovic et al., 2013), implicating them as causal mechanistic processes.

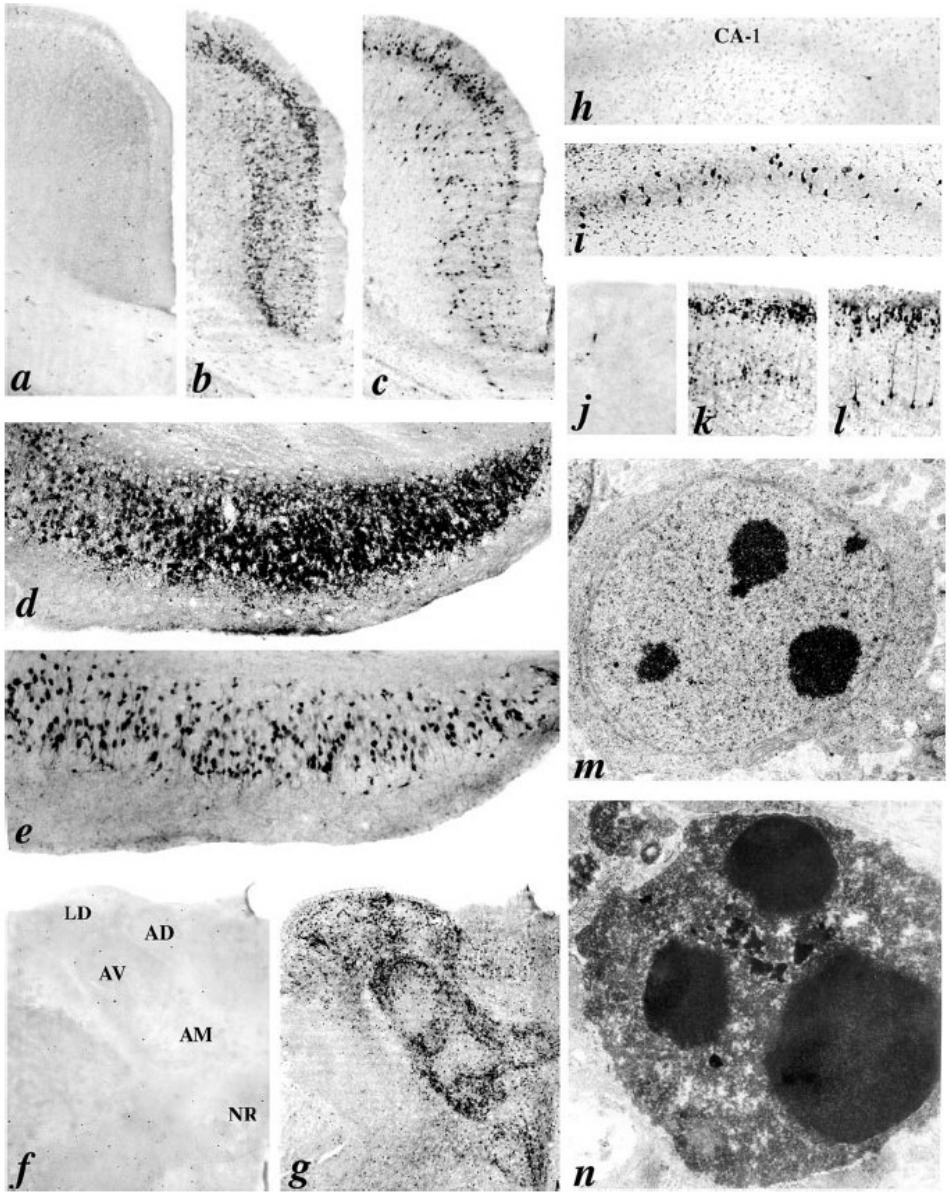

Figure 1.4: Reproduced with permission from (Jevtovic-Todorovic et al., 2003b). Photomicrographs of brain sections demonstrating neuronal apoptosis in 7 day old rat pups, following a 6 hour exposure to isoflurane, nitrous oxide plus midazolam. Subfigures a, f, h and j are from control rats and subfigures b-e, g, i, k and l are from anaesthetic exposed rats. Silver staining was used for sections a, b, d, f, g and k. Immunohistochemistry using caspase-3 activation was used for sections c, e, h-j and l. Individual nuclei shown in the anterior thalamus (f and g) are laterodorsal (LD), anterodorsal (AD), anteroventral (AV), enteromedial (AM), and nucleus reunions (NR). Sections m and n are, early stage (m) and late stage (n), electron micrographs showing the ultrastructural appearance of neurons undergoing apoptosis.

So how do these two principal effects, within the field, relate to young adults? Vulnerability to anaesthetic-induced neuronal apoptosis has been linked to the period of peak synaptogenesis in neonatal animals; and separately to processes involved in

Alzheimer's disease pathology (which it is thought provides a potential mechanism for POCD - Post Operative Cognitive Disorder - in elderly patients) (Perouansky and Hemmings, 2009, Culley et al., 2007). It is possible, however, that these factors are less relevant potential mechanisms for young healthy adults, in which case other lines of enquiry should be explored. It should be noted, for instance, that anaesthetic exposure is not invariably associated with apoptosis, particularly in young adult animals (Zhu et al., 2010).

Anaesthetic-induced neuroplasticity has been reported in association with both volatile and injectable anaesthetic agents, with alterations in the ultrastructure of surviving (non-apoptotic) neurons suggesting the induction of changes in neuronal circuitry. Briner and colleagues have demonstrated enduring, anaesthetic-induced, structural modifications of pre-frontal cortex neurons (De Roo et al., 2009, Briner et al., 2010, Briner et al., 2011), following exposure to both volatile and injectable agents. Given the association between dendritic spine dynamics and cognitive processing (Kasai et al., 2010), further investigation in young adult animals could provide information regarding the underlying neurobiological mechanisms of anaesthetic-induced cognitive impairment.

For the most part, anaesthetic-induced neurotoxicity has not been considered when investigating neuroprotection and *vice versa*. It may be, however, that anaesthetic agents can have co-occurring neuroprotective *and* neurotoxic effects. Investigating such a possibility is outside of the scope of this thesis but could have implications for both behavioural neuroscience research and human health.

Scope of the thesis

Aim 1: to better understand the behavioural phenotype of anaesthetic-induced cognitive impairment

To this end, in Chapter 3, the previously reported impairment in win-shift radial arm maze performance in rats (Culley et al., 2004b) is established to provide an anchor point from which to work. Additionally, the generality of anaesthetic-induced, short-term, spatial memory impairment is investigated, by assessing whether the same anaesthetic regimen would cause deficits in short-term, spontaneous, spatial novelty preference. The effect of anaesthesia on a different cognitive domain, that of attention (using the 5-choice serial reaction time task and the sustained attention task to assess post-anaesthetic attention as form of executive function), is investigated in Chapters 4 and 6.

Aim 2: better understand the conditions that determine the magnitude of effect or expression of the phenotype of anaesthetic-induced cognitive impairment

In Chapters 3 and 4 the relative effects of single or multiple episodes of anaesthetic exposure, on spatial working memory and tasks of attention, are compared in young adult rats. The effects of this manipulation of the anaesthetic regimen (single vs. multiple exposures) on those two cognitive domains (spatial working memory and attention) are also assessed when anaesthetic exposure occurs during development (Chapters 5 and 6). A number of different anaesthetic regimens are used throughout these chapters. For example, in Chapter 4 the effects of a volatile based anaesthetic

combination, on tasks of attention, are compared with the effects of a combination of injectable anaesthetic agents. The rationale for agent choice is given in Section 4.1 of General Methods: volatile anaesthetic regimens, and in the Materials and Methods section of Chapter 4: injectable anaesthetic regimen.

Aim 3: to better understand the neuronal mechanisms underpinning anaesthetic-induced cognitive impairment

The results from Chapters 3 and 4 were used to inform anaesthetic regimen and anatomical candidates for Chapter 7, where the effects of a single anaesthetic exposure during adulthood, on dendritic ultrastructure and spine density, are investigated.

CHAPTER 2: GENERAL METHODS

Section 1: Ethical statement

Animals were cared for and used in accordance with the principles of replacement, reduction and refinement at all times (Russell, 1959). Experimental procedures were carried out in strict accordance with local recommendations, guidelines from the Laboratory Animal Science Association (LASA, 2013) and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (National-Research-Council).

Experimental protocols were approved under the Animals Scientific Procedures Act 1986 (Chapter 3: Project Licence Number 30/2587) in the UK and the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai (Chapters 4 to 7: Protocol Number: LA-00071) in the USA.

Section 2: Subjects

2.1 Species

The loss of sentience that occurs during general anaesthesia involves the complex interactions between physiology, anatomy, biochemistry, pharmacology and psychology, that can currently only occur in a whole living vertebrate organism. Computer and bioengineering models do not yet exist for the brain systems that are being studied here. This program of work is primarily intended to facilitate the refinement of laboratory animal practice and ultimately the reduction of numbers of laboratory animals needed (by facilitating appropriate experimental design and better interpretation of scientific data). Aside from the obvious ethical implications, itwould therefore not be appropriate to carry out the proposed research using human subjects.

Rats are commonly used as a model in behavioural neuroscience experiments, and currently the most well developed anatomical coordinates and behavioural testing paradigms exist for this species. By using rats for this program of work, existing data can be expanded and the results will be of relevance to a wide audience.

2.2 Strain

Lister Hooded (Harlan, UK) and Long Evans (Taconic, USA) rats were used. Both strains are outbred and originally derived from Wistar rats. The Long-Evans is one of the most commonly used strains in behavioural neuroscience and the Lister Hooded is the UK equivalent of this strain. Strain differences were not the focus of

this program of work and these strains were chosen to ensure that experimental results had wide applicability for the behavioural neuroscience community.

2.3 Developmental stage

The primary aim of this program of work was to investigate the effect of general anaesthesia on the cognitive function of otherwise healthy adult laboratory animal subjects. Initial experiments investigated the effects of anaesthetic exposure during adulthood and were followed up with experiments investigating the effects of anaesthetic exposure during development on subsequent adult cognitive function. Procedures involving general anaesthesia, for the purposes of either model preparation or data collection, commonly occur at either of these two developmental stages and hence these stages were chosen so that results would be of relevance to a wide audience. In addition, as will be explained in later chapters, investigation of the effects of anaesthetic exposure during development is of relevance to human health.

2.4 Number of animals used

The number of animals used for the behavioural experiments here was based on work by Culley et al, who demonstrated anaesthetic-induced spatial memory impairments using 6 rats per group (Culley et al., 2004). In addition to this, the study that formed the impetus for this thesis work found an interaction between anaesthesia and surgically induced brain pathology, with 6-7 rats per group (Baxter et al., 2008). Therefore, the usual number of rats used per group in behavioural experiments here was 6.

Section 3: Housing and Husbandry

3.1 Rodent Housing and Husbandry in the United Kingdom

Rats were housed in conventional polycarbonate cages, with standard wood shaving rodent bedding (Harlan Worldwide), in same sex pairs or groups and according to Home Office Code of Practice (ASPA, 2014). In Experiment 1 of Chapter 3 surplus 'naïve' rats that had been singly housed before becoming available were used. The rats were used in accordance with the principle of reduction (to prevent wastage thereof) and remained singly housed to prevent fighting.

Automatically regulated lighting (12/12h light/dark cycle; light on at 0700) was used. Drinking water was provided ad libitum throughout all experiments. Standard rat chow (Harlan, worldwide) was provided ad libitum except when animals were food restricted for appetitively motivated behavioural tasks (See Section 3.3 food restriction protocol). Environmental enrichment consisted of cardboard tubes (one per every two rats) and nesting material (Sizzle-Nest, Datesand, UK).

3.2 Rodent Housing and Husbandry in the United States of America

Rats were housed in individually ventilated polycarbonate cages, with standard wood shaving rodent bedding, in same sex pairs or groups and according to the Guide for Care and Use of Laboratory Animals (National-Research-Council, 2011). Where applicable (Chapters 5 and 7), dams were housed (one per cage) with their litter until weaning at twenty-one days postpartum.

Automatically regulated lighting was used (12/12h light/dark cycle; light on at 1900 i.e., the opposite lighting schedule to that in the United Kingdom - this was due to limitations in Institutional facilities, was not intentional and is discussed in later chapters). Drinking water was provided ad libitum throughout all experiments. Standard rat chow was provided ad libitum except when animals were food restricted for appetitively motivated behavioural tasks (See Section 3.3 food restriction protocol). Environmental enrichment consisted of Perspex tubes (one per every two rats) and nesting material (Sizzle-Nest, Datesand International).

3.3 Food restriction protocol

Ten to fourteen days prior to the commencement of appetitively motivated behavioural testing, rats were weighed at the same time of day for three consecutive days and the values used to calculate a mean body weight. This 'free feeding weight' was then used to calculate a target weight (85% of free feeding weight). The food was restricted over 7-11 days until a stable target weight was reached. Because rats performing the 5-choice serial reaction time task (Chapter 4) were subject to an extended training and testing paradigm, target weights for those rats were corrected for growth, on a weekly basis, using vendor growth curve data (such that target weights matched 85% of age matched weights on vendor growth curve). Target weights were maintained for the duration of behavioural testing.

Section 4: Anaesthesia

4.1 Volatile and gaseous anaesthesia

(for details of the injectable anaesthetic regimen used in Chapter 4, see Materials and Methods section for that chapter)

Anaesthetised rats received a volatile anaesthetic agent carried in (vapourised within) oxygen, with or without the addition of nitrous oxide, in a five litre, temperature controlled, transparent, anaesthetic chamber. Gas flow rates were set at a total of 2.7 litres per minute, per chamber, in all experiments. This flow rate was chosen based on prior experience; and was found to be low enough to avoid excessive subject heat loss, and high enough to avoid a build up of subject exhaled carbon dioxide within the chamber. This relatively high flow rate (compared to the minute volume of an adult [approximately 300ml/min] or neonatal [approximately 1ml/min] rat) enabled changes to the concentration of anaesthetic agents within the chamber, to occur within two minutes of altering the vapouriser setting and hence facilitated greater control over the composition of chamber gas. Volatile agents were delivered by an agent specific, temperature-compensated, plenum vaporiser (isoflurane: Tech 3; sevoflurane: Penlon Sigma), as is used in clinical and research settings. Anaesthetic machines were bespoke (AW Anaesthesia Services Ltd, UK) and were comprised of components used standardly in commercially available anaesthetic machines. For anaesthetic regimen details see Table 2.1, please note that the rationale for the anaesthetic regimen used in each chapter is given in Table 2.1. It was considered that, whilst using a single anaesthetic regimen throughout the thesis, would have facilitated more straightforward between-chapter comparisons, it would also have compromised

the generalisability of results to the field by preventing the flexibility to follow changes in focus within the field.

Anaesthesia was induced with either 3% isoflurane (isoflurane regimens, IsoFlo: Abbott Animal Health, UK/USA) or 6% sevoflurane (sevoflurane regimens, SevoFlo: Abbott Animal Health, USA), carried in 100% oxygen, until loss of righting reflex and response to toe pinch; at which point the timed exposure to the chosen anaesthetic regimen began. Response to toe pinch was judged as withdrawal of the limb, muscular twitching or increase in muscle tone, and/or changes to the rate, depth or pattern of respiration i.e. motor or autonomic nervous system responses. Once anaesthetic exposure began, rats were placed in ventral recumbency for the duration of the anaesthetic period, in order to minimise compression of the lungs. Control rats were placed in an identical chamber, to those that were exposed to anaesthetic agents, and were exposed to carrier gas without either volatile or gaseous anaesthetic agents, for the same length of time and at the same total flow rate (2.7 litres per minute) as anaesthetised rats. Chamber anaesthetic agent concentration (plus nitrous oxide concentration where applicable), carbon dioxide and oxygen concentrations were monitored continuously (Vitalstore: Vetronic Services Ltd, Devon, UK).

Each episode of anaesthesia was two hours in duration. This duration was chosen, based on experience, to approximate the duration of anaesthesia required for the conduct of common behavioural neuroscience surgical procedures in rodents.

Chapter	Experiment (and behavioural task used)	Volatile agent	Gaseous agent	Carrier gas	Control condition	Rationale
3	1 (spontaneous spatial novelty preference; n=20 per group) and 2 (radial arm maze; n=6- 8 per group)	1.2% isoflurane	70% nitrous oxide	30% oxygen	Oxygen / medical air mixture, fraction of oxygen = 0.3	Reproduction of established effect (Culley et al., 2004b) Commonly used regimen in laboratory animal anaesthesia
4	1a (sustained attention task; n=8-9 per group) and 2a (5-choice serial reaction time task; n=8-9 per group) NB experiments 1b and 2b = control experiments	1.2% isoflurane	70% nitrous oxide	30% oxygen	Oxygen / medical air mixture, fraction of oxygen = 0.3	Extension of experiments in Chapter 3
	2c (5-choice serial reaction time task; n=5-7 per group)	1-3% isoflurane	none	100% oxygen	100% oxygen	Reproduction of established effect
5	1 (radial arm maze n=8-11 per group)	1.8% isoflurane	none	100% oxygen	100% oxygen	Single agent only The most commonly used volatile agent in the lab animal field
6	1 (sustained attention task; n=10 per group) and 2 (attentional set shifting; n=7-9 per group)	2.8% sevoflurane	none	Oxygen / medical air mixture, fraction of oxygen = 0.3	Brief maternal separation in oxygen / medical air mixture, fraction of oxygen = 0.3	Single agent only Of increasing interest to the lab animal field due to less tissue irritation than isoflurane
7	1 (none)	1.2% isoflurane	70% nitrous oxide	30% oxygen	Oxygen / medical air mixture, fraction of oxygen = 0.3	Extension of experiments in Chapters 3 and 4

Table 2.1 Details of, and rationale for, volatile and gaseous anaesthetic regimens (including non-anaesthetic carrier gas) chosen for this program of work. For details of the injectable regimen used in Chapter 4, see Methods section of that chapter. Volatile anaesthetic agent = a volatile liquid that produces general anaesthesia when its vapour is inhaled. Gaseous anaesthetic agent = a gas that produces sedation or general anaesthesia when inhaled, and may be used as a carrier gas for volatile anaesthetic agents. Carrier gas = a chemically non-reactive gas that carries vapourised volatile anaesthetic agent for inhalation.

4.2 Physiological monitoring during anaesthesia

Adult rats

The rat's tail was exteriorised through a port in the anaesthetic chamber. Indirect blood pressure was monitored via a tail cuff, and rectal temperature was monitored and maintained at 37°C +/- 0.5°C (Powerlab: AD Instruments, Oxford, UK). Blood oxygen saturation was monitored using a transreflectance photoplethysmographic probe placed at the base of the tail (Vetronic Services Ltd, UK), and maintained above 95%.

A blood sample (<100 microlitres) was taken from the lateral tail vein of most rats at the end of the anaesthetic period and immediately analysed (Chapter 3: ISTAT Woodley Equipment, UK; all other Chapters: Radiometer ABL80, Cleveland, USA) for pH, partial pressure of carbon dioxide and partial pressure of oxygen. Once the anaesthetic was discontinued, the chamber was flushed with oxygen and 100% oxygen administered for a minimum of 10 minutes, until the return of purposeful movement, at which point the rat was returned to it's home cage, where it was continuously observed until seen to be eating and drinking.

Neonatal rats

Pups were placed in a nest of insulating material (Gamgee Tissue, Robinson, UK) within the anaesthetic chamber. Monitor probes were passed through a port in the anaesthetic chamber. Rectal temperature was monitored (PowerLab, ADInstruments Ltd, UK) and maintained at 36.5°C +/- 0.5°C. Blood oxygen saturation was monitored using a transreflectance photoplethysmographic probe placed over the caudal body / hind limbs (Vetronic Services Ltd, UK), and maintained above 95%.

Once the anaesthetic was discontinued, the chamber was flushed with oxygen and 100% oxygen administered for a minimum of 20 minutes, until the return of purposeful movement, at which point the pup was returned to the dam. All pups were wiped over with diluted dam faeces and dried before returning to the dam, with the aim of reducing rejection. All pups were continuously monitored until seen to be suckling. No pups were rejected.

Section 5: Behavioural testing

5.1 General statement

All behavioural testing was carried out by an experimenter who was unaware of treatment group. Anaesthetised and control rats were handled for the same amount of time and in the same way. The position of home cages within the housing room was counterbalanced within group, to ensure that rats were located at different heights within holding racks and different distances from the door to the housing room. Where male and females rats were used, they were kept separately, handled using separate personal protective clothing and all equipment was cleaned with alcohol solution between sexes.

All of the behavioural tasks used are detailed here and also in the respective chapters. This duplication occurs with the aim of improving readability of the chapters. Cognitive impairment is a complex heterogenous phenomenon; multiple tasks were therefore chosen in order to examine a range of psychological processes. Some tasks were used in more than one chapter, where other variables, e.g., stage of development, were different. The chapter references are given at the beginning of each task subsection.

5.2 Spontaneous spatial novelty preference on the Y maze

This task was used in Chapter 3.

Rats were acclimated to a transparent Perspex symmetrical Y maze, by allowing them to explore freely the whole maze for fifteen minutes the day preceding anaesthesia. The maze was placed in a different spatial environment for each of the acclimation (24 hours pre-anaesthesia) and three testing (48, 72 and 96 hours post-anaesthesia) days. Each arm of the maze was 30cm long, 8cm wide, with 20cm high walls. The task consisted of two phases (Exposure and Test) and took eight minutes per rat to complete. Rats were placed at the end of a maze arm and allowed to explore two arms: 'Start arm' and 'Exposure arm' (the third arm being blocked by an opaque divider). This 'Exposure phase' lasted for five minutes and began when the rat first left the Start arm.

A rat was recorded as having left an arm if all four paws were placed outside the arm and, similarly, entry into an arm was recorded when all four paws were placed within the arm. The rat was then removed from the maze and returned to its home cage for one minute. During this time the maze was cleaned with 25% ethanol, rotated randomly 120 degrees, and the opaque divider separating the 'Novel arm' from the rest of the maze was removed. The rat was then returned to the maze for the 'Test phase', placed at the end of the Start arm and allowed to explore all three arms for two minutes (starting from the time the rat left the Start arm). Allocation of arms (Start, Exposure, and Novel) to specific spatial locations was counterbalanced within each experimental group.

The amount of time the rat spent in each of the arms and the number of entries into each arm were recorded during both the Exposure phase and the Test phase. The first arm entered (Novel or Exposure) was recorded for the first testing day (48 hours post-anaesthesia). For analysis of the test phase data, a discrimination ratio (Novel arm/(Novel + Exposure arm)) was calculated for both arm entries and time spent in the arms. Naive animals prefer the Novel arm during the Test phase (which results in a high discrimination ratio), this preference relies on extra-maze cues, and the task is dependent on hippocampal function (Sanderson et al., 2007).

5.3 Win-shift radial arm maze task (spatial working memory)

This task was used in Chapters 3 and 5.

Apparatus

A 12-arm radial arm maze was used to assess spatial working memory. Each arm of the RAM consisted of an aluminium tray, 80cm long, 14cm wide and with 3cm high walls. A well at the distal end of each arm contained a food reward odour mask (a piece of Cheerios cereal that the rats could not reach to eat, ensuring that each arm continued to smell of food even after the food reward had been collected), covered by a wire grid on top of which was placed a food reward (half a piece of Cheerios cereal). The wire grid was recessed to ensure that the food reward was not visible to the rat until the arm choice had been made. At the proximal end of each arm was a manually operated clear Perspex door that led to the central chamber of the RAM. The RAM was raised 90cm from the floor and surrounded by a curtain, upon which spatial cues were located during the testing period (but not the acclimation period).

Acclimation to maze

All rats were food restricted before RAM testing began and were maintained at 85% of ad libitum levels. For each of the three days preceding anaesthesia all rats were acclimated to the maze by allowing them to explore it freely for five continuous minutes per day and collect randomly scattered food rewards (half pieces of Cheerios cereal). Rats were placed on the maze with cage mates on the first day (with the aim of reducing anxiety and facilitating exploration) and on their own for the second and third days.

Testing procedure

A win-shift task was used in which each of the 12 arms was always baited on each session of testing, but the rewards were not replaced within a trial. Rats underwent one trial of win-shift RAM testing per day for nine days. Testing was carried out by one experimenter who was blind to anaesthetic group, and occurred between 8am and 7pm seven days per week. At the beginning of the trial rats were placed in the centre of the maze with all arm doors closed. The doors were opened and the trial began when the rat entered an arm; rats were given up to 900 seconds to complete the trial (collect all 12 food rewards). The doors were used to restrict the rat to the central platform of the maze for 5 seconds between arm choices, to prevent chaining or stereotyped motor patterns (e.g., always entering the next arm to the left, which would result in errorless performance without necessarily requiring the animal to remember which arms it had already visited).

An arm entry was counted when all four paws of the rat entered an arm (regardless of whether the food reward was collected). Errors were scored when a rat entered an

arm that it had previously entered during that trial. The RAM was cleaned with surgical spirit between each rat and rotated by 30 degrees in a pseudorandom direction (clockwise or anticlockwise) at the end of each testing day, in order to prevent the use of intra-maze cues for maze navigation.

5.4 Sustained attention task

This task was used in Chapters 4 and 6.

Apparatus

Animals were trained in sound attenuated operant chambers (Med Associates, VT, USA) equipped with two retractable levers, a house light (2.8 W), a 45 mg pellet dispenser, a 2900 Hz sonalert tone generator, and three panel lights (2.8 W). The food dispenser, panel lights, and retractable levers were all located on the same wall. The levers were located either side of the central food dispenser. The tone generator and house light were located on the opposite wall. The signals (or stimulus) used for this task were the panel lights. Records of signal presentation, lever operation, and food pellet delivery were maintained using Med-PC software (MedAssociates, VT, USA). Rats were assigned to a particular testing box for the duration of behavioural training and testing.

Lever training

All rats were food restricted before SAT training began and were maintained at 85% of ad libitum levels (see Section 3.3 for details). Training occurred between 11am and 2pm five days per week, and consisted of one 30-minute session per day. Rats were trained to lever press for food, using a fixed-ratio-one schedule (receiving a

food reward for each lever press) until they produced at least 50 responses on each of the two levers, for two consecutive days, at which point task training began.

Initially food was placed on the levers to encourage the rats to interact with them. Once rats started to lever press they were rewarded for each press, regardless of which lever was pressed. There was no limit on the number of times a rat could lever press during the 30-minute training session. During this period of lever press training a one-off correction schedule was used if rats developed a side bias (preference for a particular lever). A side bias was defined as pressing either the right or the left lever for more than 70% of the total presses within a session, for more than two consecutive sessions). The correction schedule was a 30-minute schedule (which replaced the following day's session) during which rats were rewarded for pressing their least preferred lever and not rewarded for pressing their preferred lever.

Task training

Rats were food rewarded for distinguishing between signal (central panel and left panel illumination for 1 second) and non-signal (absence of visual stimulus) trials by pressing the appropriate one of two levers extended 2 seconds after presentation (or lack of presentation) of the stimulus. Rats were required to press the left lever on signal trials and the right lever on non-signal trials. The levers remained in place until pressing occurred, for up to a maximum of 4 seconds. The left and right lever function was not counterbalanced, i.e., all rats, in all trials, were required to press the left lever on signal trials and the right lever on non-signal trials.

Correct lever presses were defined as *hits* when they occurred, at the left lever, on a signal trial and *correct rejections* when they occurred, at the right lever, on a non-

signal trial. Incorrect lever presses were defined as *misses* when they occurred on a signal trial and *false alarms* when they occurred on a non-signal trial. If the animal failed to respond or responded incorrectly, the levers were retracted, the inter-trial interval (ITI) (12 +/- 3s) was reinstated and the trial was repeated up to three times (correction trials). If the animal failed to respond or responded incorrectly after three correction trials a forced-choice trial occurred. During a forced-choice trial the event (signal or non-signal) was repeated but only the correct lever was extended and remained active for up to 90 seconds (with illumination of the central and left panel if on a signal trial). These forced-choice trials served to prevent development of a side bias during this phase of training.

Shaping

Once rats responded correctly to at least 70% of signal and non-signal events for at least three consecutive days, the signal was changed to one second illumination of the central panel light only (rather than central plus left panel). Once rats responded correctly to at least 75% of signal and non-signal events for at least three consecutive days, rats began training to criterion with variable signal length.

The length of the signal was modified to 500, 50 or 25 milliseconds. Rats performed for a maximum of 162 consecutive trials or 40 minutes (one session) per day. Each session was divided into three blocks of 54 trials, each counterbalanced for signal length, such that each block consisted of 9 trials of each signal length and 27 non-signal trials. Rats were trained to a criterion of >75% hits to 500ms signals and >75% correct rejections to non-signal trials, for at least two consecutive sessions. Correction

trials and forced-choice trials were not used during this phase. Once all rats reached criterion, baseline performance was assessed on the day preceding anaesthesia.

Testing procedure

Post-anaesthetic testing consisted of one session per day for six days. Rats performed for a maximum of 162 consecutive trials or 40 minutes per day. There was no difference between these sessions and the final shaping schedule. Signal duration varied (as in the final shaping schedule), and all trials were presented in a temporally unpredictable fashion to heighten attentional demands.

During each session the number of hits (correct signal-trial lever presses), misses (incorrect signal-trial lever presses), correct rejections (correct non-signal-trial lever presses), false alarms (incorrect non-signal-trial lever presses) and errors of omission (lack of lever press) were recorded.

Once rats reached criterion, they were allocated to anaesthetic groups, counter-balanced for sex (Chapter 6 only), level of performance (relative number of hits [hits / hits + misses] and the relative number of correct rejections [correct rejections / correct rejections + false alarms]), and as far as possible for body weight. Rats then underwent one day of baseline testing before being anaesthetised.

Behavioural performance measures recorded included signal detection (a percent correct measure: number of hits / number of hits plus number of misses multiplied by 100), correct rejections (a percent correct measure: number of correct rejections / number of correct rejections plus number of false alarms multiplied by 100) and the number of omissions for signal and non-signal trials separately.

5.5 5-choice serial reaction time task

This task was used in Chapter 4.

Apparatus

Rats were trained in Bussey-Saskida Rat Touch Screen Chambers (Model 80604, Lafayette Instruments, IN, USA) using ABET II Software for Touch Screen (Model 89505). Sound-attenuating operant boxes were fitted with a trapezoidal enclosure 33 cm long, 31 cm wide and 30 cm high, covered with a clear acrylic sheet during testing. The large end of the trapezoidal enclosure was comprised of a touchscreen, covered by a black acrylic matt with five 3 x 3 cm response windows cut out in a horizontal plane and at a height just above the rats' eye level. The opposite end of the enclosure contained a food magazine into which food pellets were dropped from an automated dispenser. The magazine was equipped with a light and an infrared beam-break. The chamber also contained a house light and a small speaker. Each rat was assigned to a particular testing box for the duration of behavioural training and testing.

Nose poke training

For a detailed description of the task see (Bari et al., 2008).All rats were food restricted before 5CSRTT training began and were maintained at 85% of ad libitum levels (see Section 3.3, Chapter 2 - General Methods for details). Training occurred between 1100h and 1400h five days per week. Rats were trained to make nose-pokes in any one of five reward windows, using a fixed-ratio-one (FR1) schedule (receiving a food reward for each nose poke). Initially food was placed in each of the response windows to encourage rats to interact with them. Once rats started to nose poke they were rewarded for each poke, regardless of which window was poked. During this

phase of nose poke training a one-off correction schedule was used if rats failed to poke a particular window(s). The correction schedule was an additional daily session where only nose pokes at particular windows were rewarded. Once rats were reliably poking each of the 5 windows (decided subjectively by the experimenter via video camera) task training began.

Task training

Rats were required to sustain visual spatial attention (visual scanning) of the five response windows. A brief visual stimulus (illumination) occurred in a pseudo-random location (counterbalanced within a session to ensure equal numbers of illuminations at each of the five response windows), and the rat was required to nose poke that location within a limited time (limited hold: LH). Correct trials were rewarded with a pellet delivered to the food magazine; a nose-poke in the food magazine was required to initiate new trials, and all trials were separated by an inter-trial interval (ITI), which was 5 seconds long during training and began after the nose-poke in the food magazine. Incorrect responses were punished with a 30s time-out period with the house light on. Trials in which the animals made premature responses or failed to make a response were similarly punished. Initially training sessions consisted of 100 trials in up to 30 minutes. The stimulus duration (illumination) was 30 seconds and the LH (the time allowed between stimulus presentation and nose poke) was 30 seconds. Once the rat reached a pre-determined criterion, the training session parameters were altered to decrease the stimulus duration and LH, and thereby increase the attentional demands. For a list of the training session stages and criterion required see Table 4.3.

Training stage	Stimulus duration (s)	Inter trial interval (ITI) (s)	Limited hold (LH) (s)	Criterion to move to next stage
1	30	5	30	≥ 30 correct trials
2	20	5	20	≥ 30 correct trials
3	10	5	10	≥ 50 correct trials
4	5	5	5	≥ 50 correct trials > 80% correct
5	2.5	5	5	≥ 50 correct trials > 80% correct < 20 % omissions
6	1.25	5	5	≥ 50 correct trials > 80% correct < 20 % omissions
7	1	5	5	≥ 50 correct trials > 80% correct < 20 % omissions
8	0.9	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
9	0.8	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
10	0.7	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
11	0.6	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
12	0.5	5	5	≥ 75 correct trials (of 150 total) > 75% correct < 20 % omissions
13	0.5	5 for first 50 trials 2 for middle 50 trials 5 for last 50 trials	5	≥ 75 correct trials (of 150 total) > 75% correct < 20 % omissions

Table 4.3 Overview of steps involved in the training of rats on the 5-choice serial reaction time task. Following nose poke training, training proceeds via 13 distinct criterion-based stages. Stages 1-11 comprise 100 trials in 30 minutes and stages 12 and 13 comprise 150 trials in 45 minutes. Table adapted from (Bari et al., 2008).

Rats were trained to criterion, for five consecutive days, on training stage 13 (>75% accuracy, <20% omissions, maximum 150 trials or 45 minutes of testing) for a maximum of 150 consecutive trials or 45 minutes. This final version of the task included the first and last 50 trials having an ITI of 5s, and the middle 50 trials having an ITI of 2s. The purpose of this increased event rate (number of trials per minute) for the middle 50 trials was to increase the attentional demands of the task (see Fig 4.1 for a schematic representation of the task). After task training was

complete rats were allocated to anaesthetic groups, counter-balanced for level of performance (percentage accuracy: correct choices / correct plus incorrect choices, multiplied by 100) and as far as possible for body weight, and pre-anaesthetic testing began. Pre-anaesthetic (baseline) testing consisted of six days of testing, the last of which occurred 24hrs before anaesthesia or control condition. Post-anaesthesia testing occurred 24hrs after anaesthesia and consisted of six days of testing.

Behavioural measures included choice accuracy (a percent correct measure: number of correct responses / correct responses plus incorrect responses, multiplied by 100), omissions (a percent omission measure: number of omitted trials / total number of trials, multiplied by 100), number of premature responses (nose pokes that precede stimulus), number of perserverative responses (repeated nose pokes at the correct response window after a stimulus), the response latency (time taken to respond to a stimulus) and the reward collection latency (time taken to collect food reward).

5.6 Attentional set shifting task

This task was used in Chapter 6.

Apparatus

Attentional set shifting performance was tested using terra-cotta pots that rats were taught to dig in to receive a food reward. The pots were 10cm high with an internal diameter of 10.2cm. The inside of the pots were filled with digging media which were scented using diluted aromatherapy oils (essential oils diluted 1:100 in vegetable oil). The outside of the pots were covered with textures. Each pot therefore contained up to three stimulus types (digging media, odour and texture). Pot(s) were placed into one end of a rectangular plastic testing box (91.4cm long, 45.7cm wide and 25.4cm high). A removable divider separated the end of the box that contained the pot(s) and a holding area (30cm long) where the rat was held prior to initiation of the trial. The purpose of the removable divider was to control precisely the onset of the task, without the potential bias of placing the rat into the box after the pot(s) had been placed.

Food reward for all stages of training and testing was a quarter piece of Cheerios breakfast cereal, and the unabated pot always contained an equal but crushed amount of food reward to act as an odour mask, and prevent the rat from using the scent of the cereal to identify the correct stimulus.

Shaping (day 1)

The rat was placed in the holding area of the testing box whilst an unscented pot filled with rodent bedding material, with a food reward placed on top of the bedding, was placed at the other end of the box. The divider was removed and a timer begun. The

rat was then allowed 90s to retrieve the food reward. Once the food reward was collected or at 90s, the pot was removed from the box and the rat returned to the holding area. The procedure was then repeated and the position of the pot was pseudorandomly varied from the right and left side of the box to prevent development of a response side bias. Once the rat had retrieved 10 unburied food rewards, the food reward was buried at increasing depths until the rat completed ten consecutive trials using the forelimb to dig for a fully buried reward.

Exemplar training (day 2)

Rats were given a series of discriminations to perform, in order to introduce the types of stimuli that would be tested in the IDED task (digging media, odour and texture). For this stage, 2 pots were placed in the box. Both pots contained one stimulus type e.g., odour. Two different exemplars of the stimulus (e.g., for odour, one pot would be scented with pine and the other with black cherry) were used and the rat was consistently rewarded for digging in the same one of the two exemplars. Each rat was required to reach a criterion of six consecutive correct choices for each of the three stimulus types (dimensions).

Exemplars were as follows, digging media: shredded green tissue paper vs shredded white tissue paper; odour: pine vs black cherry; texture: white Vetbed (faux sheep skin) vs the reverse side of the Vetbed. For odour and texture discriminations, both pots were filled with shredded cardboard to hide the reinforcer.

Intra-dimensional / extra-dimensional attentional set shifting task (day 3)

IDED testing consisted of the following phases in the following order: the simple discrimination (SD), compound discrimination (CD), compound reversal (CDR), intra-dimensional shift (ID), intra-dimensional shift reversal (IDS), extra-dimensional shift (ED) and extra-dimensional shift reversal (EDS). For each of these phases the rat was allowed four discovery trials, where the rat had 90 s to explore both pots. If the rat made an incorrect choice (dug in the wrong pot), an incorrect choice was recorded but the rat was allowed to search in the correct pot and retrieve the food reward. Rats were allowed to dig until the food reward was collected following all correct choices. Choice latency (time between removal of the divider and first digging behaviour - in either the correct or incorrect pot) was recorded and during non- discovery trials rats were allowed up to 60 s to make a response. If either an incorrect response was made or 60 s expired (in which case an omission was recorded) the trial was aborted and the rat returned to the holding area. Trials continued for each phase until the criterion level of six consecutive correct responses was made. The number of trials taken to reach criterion, for each phase, was recorded.

Simple discrimination

The simple discriminations tested between 2 pots that differed on only one dimension (stimulus type - digging media, odour or texture). For example, in a test of odour discrimination a pot scented with cinnamon was baited with a food reward and presented in combination with a pot scented with patchouli. Digging media and texture were identical for both pots. Alternate pairs of pots were tested where one dimension, e.g., digging media, varied between pairs. For example, cinnamon / light foam shapes vs patchouli light foam shapes would alternate with cinnamon / dark foam

shapes vs patchouli / dark foam shapes, and the pot containing cinnamon was rewarded in each case, regardless of the digging media type. In this way rats would learn that the digging media were irrelevant.

Compound discrimination

In the compound discriminations, the rewarded stimulus remained constant (as in the simple discriminations), but the pots now differed on two dimensions rather than one. Alternate testing pairs would be, e.g., cinnamon / light foam shapes vs patchouli / dark foam shapes and cinnamon / dark foam shapes vs patchouli / light foam shapes, where the pot containing cinnamon was rewarded in each case, regardless of the digging media type. In this example, rats would therefore have to ignore digging media (as in the simple discriminations) but would be presented with 2 pots that differed in two dimensions (odour and digging media) not just the relevant one (odour).

Compound discrimination reversal

For the reversal trials the rat was reinforced for responding to the previously unrewarded exemplar of a dimension, e.g., patchouli odour. Otherwise, the testing pairs were identical to those used in the compound discriminations.

Intra-dimensional shift

A complete new set of stimuli was introduced in the ID phase. However, the same dimension that predicted reward in the SD, CD and CDR (e.g., odour) predicted reward in the ID. Rats were therefore required to shift attention to a novel stimulus within the same dimension as previous phases, i.e., make an intra-dimensional shift in attention.

Intra-dimensional shift reversal

For the IDS reversal trials the rat was reinforced for responding to the previously unrewarded exemplar of a dimension. Otherwise, the testing pairs were identical to those used in the intra-dimensional discriminations.

Extra-dimensional shift

In the extra-dimensional shift the previously irrelevant dimension, e.g., digging media, now predicted reward. Rats were therefore required to shift attention to a stimulus within a different dimension, i.e., make an extra-dimensional shift in attention.

Extra-dimensional shift reversal

For the EDS reversal trials the rat was reinforced for responding to the previously unrewarded exemplar of a dimension. Otherwise, the testing pairs were identical to those used in the extra-dimensional discriminations.

There were six possible sequences of shifts between dimensions in the ID and ED phases (digging media to texture, odour to digging media, texture to digging media, odour to texture, digging media to odour, texture to odour). The first four of these possible sequences were used and rats were pseudorandomly assigned a sequence of testing, balanced as far as possible for prior anaesthesia exposure and sex.

Behavioural measures recorded during each of the phases were: the number of trials taken to reach criterion of six consecutive correct choices (trials to criterion); the time

taken to make a correct choice (correct choice latency) and the time taken to make an incorrect choice (incorrect choice latency).

CHAPTER 3: ISOFLURANE PLUS NITROUS OXIDE ANAESTHESIA IMPAIRS WIN-SHIFT RADIAL ARM MAZE PERFORMANCE BUT NOT SPATIAL NOVELTY PREFERENCE

Introduction

Post-operative cognitive dysfunction (POCD) refers to a subtle dysfunction of one or more cognitive domains following surgery, of which memory is typically affected. It is well established that anaesthesia has short term effects on cognition (for the duration of anaesthetic administration) but an increasing body of work has shown that anaesthesia also has longer term effects on learning and memory beyond the point at which the drugs are excreted from the body, thereby implicating anaesthesia as a causal factor for POCD (Moller et al., 1998, Zywiel et al., 2014, Jevtovic-Todorovic et al., 2013).

Data from in-vivo animal experiments regarding the presence of POCD following anaesthesia are compelling. Cognitive impairments have been demonstrated, in both male and female rodents following exposure in adulthood to commonly used anaesthetic agents (Culley et al., 2004b, Wiklund et al., 2009, Bianchi et al., 2008, Le Freche et al., 2012, Lin and Zuo, 2011, Saab et al., 2010, Zurek et al., 2012). Studies have been controlled for physiological derangement such that results are not confounded by the hypotension, hypoxia or hypercapnia that can accompany rodent anaesthesia in some circumstances.

For example, adult rats exposed to two hours of isoflurane plus nitrous oxide anaesthesia demonstrate impaired performance on a win-shift radial arm maze (RAM) task of short-term spatial working memory, when testing begins forty eight hours post-anaesthesia and continues for twenty one days (Culley et al., 2004b). In a more recent study, adult rats exposed to two hours of isoflurane alone were impaired on contextual fear conditioning, when testing began two weeks after exposure. Behavioural impairments were associated with increases in hippocampal inflammatory cytokines and decreases in neuronal density within the hippocampus (Lin and Zuo, 2011). Contextual fear conditioning (as well as the win-shift RAM task, used by Culley) is dependent on hippocampal function, i.e., performance of the behavioural task is affected by hippocampal lesions), and Lin et al proposed that the changes found within the hippocampus provide a possible mechanism for POCD.

The combination of human and animal data suggest that anaesthetic exposure can disrupt subsequent cognitive function, and there is therefore a need to develop robust behavioural animal models to permit the characterisation of anaesthetic-induced POCD following exposure in adulthood. Phenotypic characterisation is essential for the identification of the psychological processes involved, and ultimately therefore for the determination of neurobiological mechanisms. Such an approach also informs the design of prospective clinical patient studies (so that particular patient groups can be identified and appropriately specific and sensitive behavioural tasks selected) as well as the design of animal behavioural studies, where anaesthesia may act as a confound.

Indeed, anaesthetic-induced cognitive impairment is not only of direct clinical importance; but also has implications for the validity of data produced from *in-vivo* pre-

clinical experiments. Of the 4 or so million animals used in biomedical research each year in the UK alone, approximately a third undergo anaesthesia as part of experimental protocols (Statistics, 2013). Anaesthesia is used increasingly for restraint purposes, for example for imaging procedures, as well as to facilitate painful or invasive procedures, and some animals undergo multiple anaesthetics throughout their experimental life. Whether anaesthesia itself is a critical variable for neuroscience experiments is therefore a question fundamental to the interpretation of pre-clinical data, regardless of how subtle or transient the effects might be.

The generality of the behavioural effect demonstrated by Culley et al was investigated here by testing whether the same anaesthetic regimen would cause deficits in performance of a different spatial memory task - the spontaneous spatial novelty preference (SSNP) task. This task utilises a transparent Y maze to assess rapidly acquired, short-term spatial memory, and relies on the fact that normal rats prefer a novel to a familiar (recently visited) environment. Like the RAM win-shift task, the SSNP task is hippocampal dependent and relies on the use of visual extra-maze cues (Sanderson et al., 2007). Unlike the RAM successful performance relies on spontaneous exploratory behaviour within a trial, rather than the memory of associations across trials, and is therefore inherently variable in normal healthy subjects. However, it is much quicker to complete than the RAM (there is no learning or practice effect across trials) and does not involve appetitive motivation, as it relies on spontaneous exploration. The SSNP task avoids the need for food restriction, the stress of escape from deep water or electric shock that the RAM, Morris water maze and fear conditioning paradigms (respectively) involve. It therefore potentially provides a fast, refined method for detecting, and further investigating, the hippocampal-dependent memory deficits that have been demonstrated using these

other tasks. It was hypothesised that the SSNP performance of previously anaesthetised rats would be impaired compared to non-anaesthetised control rats.

In a second experiment, the deficit in spatial win-shift RAM performance reported by Culley and colleagues, was reproduced and extended to investigate the effect of repeated anaesthetic exposure. The RAM win-shift task (see Section 5.2, Chapter 2 - General Methods) is a hippocampal-dependent task, where rats use visual extra-maze cues to avoid visiting previously rewarded 'winning' arms of the RAM, and thus 'shift' arm choice to unvisited locations. Reproduction of the deficit in win-shift performance, reported by Culley and colleagues, was intended partly to provide a positive control for the effect of isoflurane plus nitrous oxide exposure during adulthood on SSNP performance. Furthermore, in order to extend the characterisation of the phenotype of anaesthetic-induced cognitive impairment following exposure in adulthood, the effect of repeat episodes of anaesthesia was also investigated. The performance of rats that received a single anaesthetic exposure was compared to those that received three anaesthetic exposures. Each exposure was separated by one week, in order to ensure full recovery between anaesthetics and to match the interval standardly advised for *in-vivo* experiments in the UK (Principal- Home-Office-Inspector, 2008).

Mice exposed to repeat episodes of isoflurane (Bianchi et al., 2008) or sevoflurane (Le Freche et al., 2012) show impaired spatial memory performance on the Morris water maze. However, neither study included a single exposed group and so whether repeated exposures increase the magnitude of the phenotype associated with anaesthesia in adulthood, is not known. Evidence from human studies suggests that children who receive repeated general anaesthetics before the age of four years, have

a greater rate of learning disability when compared to those who receive a single exposure (Wilder et al., 2009); demonstrating that repeated episodes of anaesthesia may be more detrimental than a single episode. It was therefore hypothesised that the same anaesthetic protocol used by Culley et al, given repeatedly to adult rats, would be associated with a greater impairment in RAM performance.

Materials and Methods

Subjects

Male Lister Hooded rats (Harlan UK), 3.5 months old (307-363g) at the time of anaesthesia were singly housed[11] (Experiment 1) or group housed (Experiment 2), in polycarbonate cages with automatically regulated lighting (12/12h light/dark cycle, lights on at 0700).

Experimental design

Experiment 1: Spontaneous spatial novelty preference on the Y maze

Rats were randomly allocated to one of two groups: control condition (n=20) and one 2 hour period of anaesthesia (n=20), counterbalanced for body weight.

Innate preference for spatial novelty, during spontaneous exploration, was tested (see below for details). Animals underwent one trial of SSNP testing, at each of the following time points: 48, 72 and 96 hours post-anaesthesia. Anaesthetic-induced cognitive impairment is thought to be transient in adults (Crosby et al., 2005) but the time point of peak impairment is not known. SSNP trials at different time points (one per day for three days) were therefore conducted in order to avoid the likelihood that

[1] Rats for Experiment 1 were surplus naive rats from an unrelated experiment and had been singly housed prior to becoming available. They were used for Experiment 1 in order to prevent unnecessary wastage (in line with the principle of reduction) and were not re-paired due to the risk of fighting (in line with the Home Office Code of Practice for Housing and Husbandry).

impairment would remain undetected in the immediate post-anaesthetic period. Each of the three trials were carried out in different spatial environments (separate rooms) to ensure that the extra-maze visual spatial cues were distinct on each of the testing days (Fig 3.1).

Experiment 2: Win-shift on the radial arm maze

Rats were food restricted (see Section 3.3 for detailed methods) and randomly assigned to one of three groups: control condition (Group 0A; n=8); one 2 hour period of anaesthesia administered 48hrs before behavioural testing began (Group 1A; n=8); or three 2 hour periods of anaesthesia, each separated by one week, the last of which was administered 48 hours before behavioural testing began (Group 3A; n=6).

Spatial short-term memory (win-shift) performance was tested on a 12-arm RAM, where each of the 12 arms was baited and food rewards were not replaced within trials. This task is commonly referred to as a working memory task. Animals underwent one trial, of RAM testing per day, for nine days post-anaesthesia (Fig 3.1).

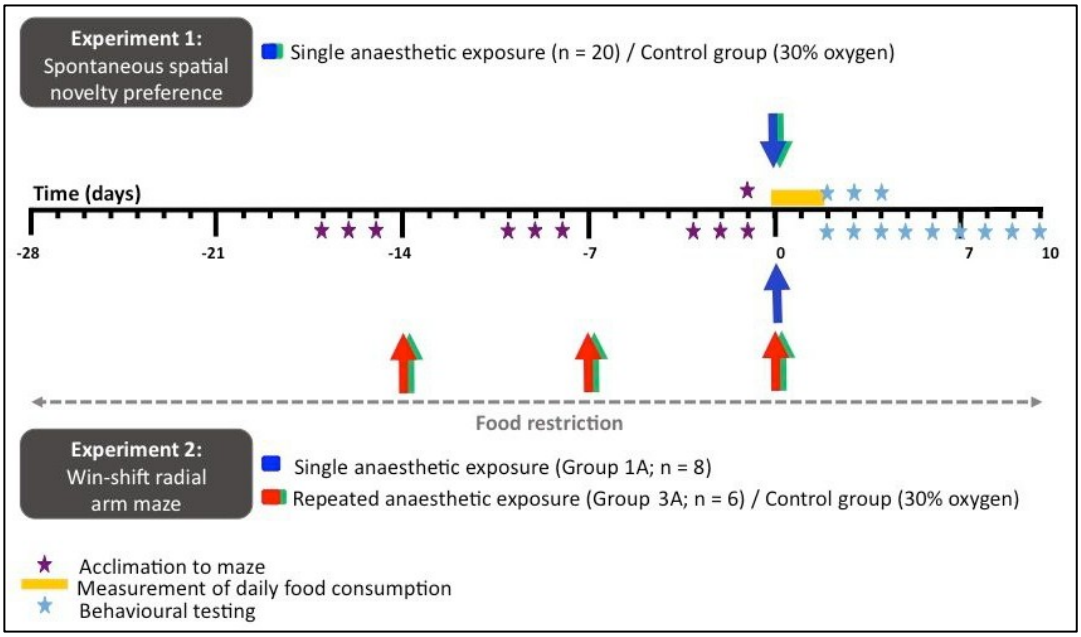

Fig 3.1: Timeline representing the experimental design for Experiments 1 and 2. In Experiment 1 (above the timeline) rats were acclimated to a Y maze (purple asterisks) and then assigned to either receive a single anaesthetic exposure (dark blue arrow) or control condition (green shadow) at day 0. A task of spontaneous spatial novelty preference was conducted at 48, 72 and 96 hours post-anaesthesia (light blue astericks). In Experiment 2 (below the timeline) rats were food restricted (grey interrupted double arrow) and then assigned to receive either a single anaesthetic exposure (dark blue arrow) (at day 0), repeated anaesthetic exposure (red arrows) (the last of which occurred at day 0), or the control condition (green shadow). Rats were acclimated to a 12-arm radial arm maze before each period of behavioural testing (purple asterisks). A win-shift radial arm maze task was conducted daily for nine days, from 48 hours, post- anaesthesia (light blue asterisks). Daily food consumption was measured for the first 48hrs post-anaesthesia, in rats from Experiment 1 (yellow line) - these data were used to assess food consumption post isoflurane plus nitrous oxide anaesthesia, in order to determine if effects would confound performance of the appetitively motivated task in Experiment 2.

Experimental conduct

One potential confound of appetitively-motivated tasks such as the RAM (used in Experiment 2), is the effect of anaesthesia on appetite. For instance, post-anaesthetic nausea, which is known to occur in humans and other animal species (Gan, 2006, Santos et al., 2011), may lead to a decrease in appetite, which may therefore reduce motivation to collect food reward. Experiment 1 (which did not involve food reward) provided an opportunity to investigate the effect of isoflurane plus nitrous oxide on post-anaesthetic food consumption (as an indirect measure of appetite). Rats in

Experiment 1 did not need to be food controlled and were singly housed, and could therefore have post-anaesthetic food consumption measured. Therefore, on the evening of the first and second post anaesthetic days, 40g of standard rat chow was given to each rat (anaesthetised and control rats) (Fig 3.1). The following morning the uneaten food (food remaining in the cage) for each rat was measured and used to calculate the overnight food consumption (40g minus weight of food remaining) for 24 and 48hrs post-anaesthesia.

Anaesthesia and control condition

Episodes of anaesthesia consisted of 2 hours of 1.2% isoflurane plus 70% nitrous oxide, 30% oxygen. Indirect blood pressure was recorded after one and two hours of anaesthesia (i.e. two samples per animal per anaesthetic exposure). In addition, at the end of the anaesthetic episode, a venous blood sample was taken and immediately analysed for pH, pCO_2 and pO_2. The control condition consisted of 2 hours of medical air plus oxygen (fraction of inspired oxygen 0.3), in an identical chamber with identical gas flow rate (see Section 4, Chapter 2 - General Methods for detailed methods).

Behavioural testing

Experiment 1: Spontaneous spatial novelty preference on the Y maze

Rats were acclimated to a transparent Perspex symmetrical Y maze, by allowing them to explore freely the whole maze for fifteen minutes the day preceding anaesthesia. The maze was placed in a different spatial environment for each of the acclimation (24 hours pre-anaesthesia) and three testing (48, 72 and 96 hours post-anaesthesia) days. Each arm of the maze was 30cm long, 8cm wide, with 20cm high walls. The task consisted of two phases (Exposure and Test) and took eight minutes per rat to complete (Fig 3.2). Rats were placed at the end of a maze arm and allowed to explore two arms: 'Start arm' and 'Exposure arm' (the third arm being blocked by an opaque divider). This 'Exposure phase' lasted for five minutes and began when the rat first left the Start arm.

A rat was recorded as having left an arm if all four paws were placed outside the arm and, similarly, entry into an arm was recorded when all four paws were placed within the arm. The rat was then removed from the maze and returned to its home cage for one minute. During this time the maze was cleaned with 25% ethanol, rotated randomly 120 degrees, and the opaque divider separating the 'Novel arm' from the rest of the maze was removed. The rat was then returned to the maze for the 'Test phase', placed at the end of the Start arm and allowed to explore all three arms for two minutes (starting from the time the rat left the Start arm). Allocation of arms (Start, Exposure, and Novel) to specific spatial locations was counterbalanced within each experimental group.

The amount of time the rat spent in each of the arms and the number of entries into each arm were recorded during both the Exposure phase and the Test phase. The first arm entered (Novel or Exposure) was recorded for the first testing day (48 hours post-anaesthesia). For analysis of the test phase data, a discrimination ratio (Novel arm/(Novel + Exposure arm)) was calculated for both arm entries and time spent in the arms. Naive animals prefer the Novel arm during the Test phase (which results in a high discrimination ratio), this preference relies on extra-maze cues, and the task is dependent on hippocampal function (Sanderson et al., 2007).

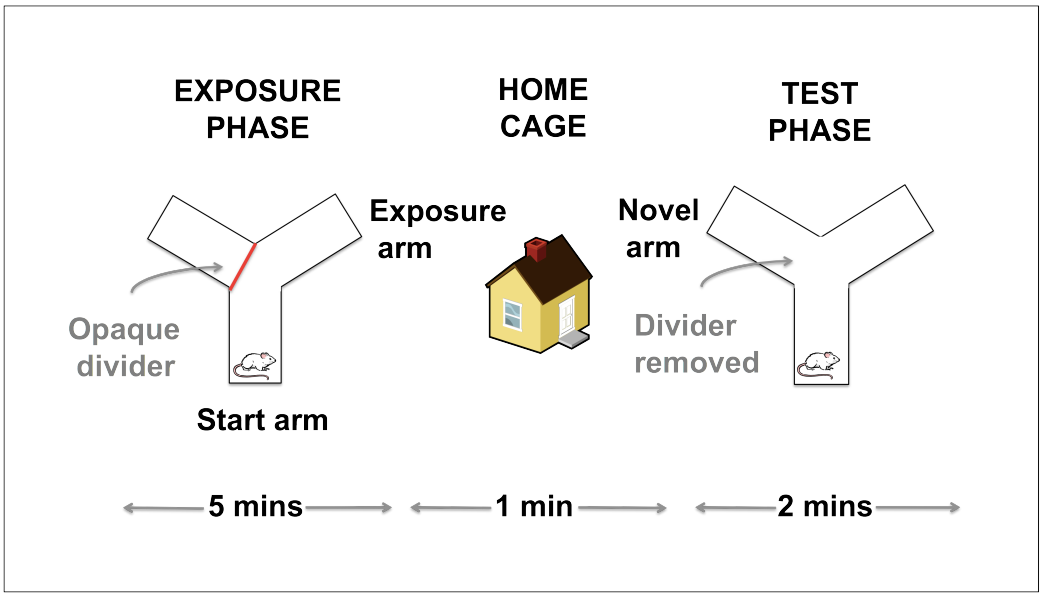

Fig 3.2: Spontaneous spatial novelty preference task. The rat is placed in the Start arm of the maze and allowed to explore two arms: 'Start arm' and 'Exposure arm' (the third being blocked by an opaque divider). This 'Exposure phase' lasts for five minutes. The rat is then removed from the maze and returned to its home cage for one minute. During this time the solid divider separating the 'Novel arm' from the rest of the maze is removed. The rat is then returned to the maze for the 'Test phase', placed at the end of the Start arm and allowed to explore all three arms for two minutes (starting from the time the rat leaves the Start arm). Control rats spend more time in, and make more entries into, the Novel arm compared to the familiar arms, during the test phase.

Experiment 2: Win-shift on the radial arm maze

A 12-arm radial arm maze was used to assess spatial working memory. Each arm of the RAM consisted of an aluminium tray, 80cm long, 14cm wide and with 3cm high walls. A well at the distal end of each arm contained a food reward odour mask (a piece of Cheerios cereal that the rats could not reach to eat, ensuring that each arm continued to smell of food even after the food reward had been collected), covered by a wire grid on top of which was placed a food reward (half a piece of Cheerios cereal). The wire grid was recessed to ensure that the food reward was not visible to the rat until the arm choice had been made. At the proximal end of each arm was a manually operated clear Perspex door that led to the central chamber of the RAM. The RAM was raised 90cm from the floor and surrounded by a curtain, upon which spatial cues were located during the testing period (but not the acclimation period).

All rats were food restricted before RAM testing began and were maintained at 85% of ad libitum levels. For each of the three days preceding anaesthesia all rats were acclimated to the maze by allowing them to explore it freely for five continuous minutes per day and collect randomly scattered food rewards (half pieces of Cheerios cereal). Rats were placed on the maze with cage mates on the first day (with the aim of reducing anxiety and facilitating exploration) and on their own for the second and third days.

A win-shift task was used in which each of the 12 arms was always baited on each session of testing, but the rewards were not replaced within a trial. Rats underwent one trial of win-shift RAM testing per day for nine days. Testing was carried out by one experimenter who was blind to anaesthetic group, and occurred between 8am and

7pm seven days per week. At the beginning of the trial rats were placed in the centre of the maze with all arm doors closed. The doors were opened and the trial began when the rat entered an arm; rats were given up to 900 seconds to complete the trial (collect all 12 food rewards). The doors were used to restrict the rat to the central platform of the maze for 5 seconds between arm choices, to prevent chaining or stereotyped motor patterns (e.g., always entering the next arm to the left, which would result in errorless performance without necessarily requiring the animal to remember which arms it had already visited).

An arm entry was counted when all four paws of the rat entered an arm (regardless of whether the food reward was collected). Errors were scored when a rat entered an arm that it had previously entered during that trial. The RAM was cleaned with surgical spirit between each rat and rotated by 30 degrees in a pseudorandom direction (clockwise or anticlockwise) at the end of each testing day, in order to prevent the use of intra-maze cues for maze navigation.

The total number of arm entries per trial was recorded along with the time taken to complete the maze (up to a maximum 900 seconds). The number of errors made within the first 12 choices and the number of choices made before the first error, within a testing trial, were recorded.

Statistical analysis

Experiment 1: Spontaneous spatial novelty preference on the Y maze

SSNP behavioural parameters (i.e., number of arm entries, time spent in arms and discrimination ratios) were compared using repeated measures ANOVA, with day of testing as within-subjects factor (3 levels) and anaesthesia group as between-subjects factor (2 levels), with Fisher's LSD post-hoc analysis. The first arm choice (either Novel or Exposure) for rats in each group (anaesthesia and control) were compared using Fisher's exact test.

Experiment 2: Win-shift on the radial arm maze

Anaesthetic physiology

Blood pressure data collected from Groups 1A and 3A during the third period of anaesthesia (the period of anaesthesia just prior to behavioural testing) were compared using repeated measures ANOVA, with sample (2 levels: 1hr post-induction and 2hr post-induction of anaesthesia) as the within-subjects factor and anaesthesia group (2 levels) as the between-subjects factor. In addition, blood pressure data from Group 3A were compared using repeated measures ANOVA, with anaesthetic exposure phase (3 levels: 1st, 2nd and 3rd anaesthetic exposure) and sample (2 levels: 1hr and 2hr post-induction) as the within-subjects factors.

Due to a technical problem, blood gas samples from Groups 1A and 3A during the third period of anaesthesia (as well as one sample from Group 3A during the 2nd anaesthetic phase) were not analysed. Blood gas data collected from Group 3A during the first and second period of anaesthesia were compared using paired sample t-test.

Behavioural testing

Win-shift RAM behavioural parameters (i.e., the total number of arm entries, time to complete the maze, number of errors within the first 12 choices and the number of choice before the first error occurred) were compared using repeated measures ANOVA, with day of testing as the within-subjects factor (9 levels) and anaesthesia group as the between-subjects factor (3 levels), with Fisher's LSD post-hoc analysis.

Experimental conduct

Food consumption data were compared using repeated measures analysis of variance (ANOVA) with post-anaesthetic day as the within-subjects factor (2 levels: 1st and 2nd 24 hour period after anaesthesia), and anaesthesia group as the between-subjects factor (2 levels).

Results

Experiment 1: Spontaneous spatial novelty preference on the Y maze

Anaesthetic physiology

Body temperature was successfully maintained between 36.5°C and 37.5°C in all anaesthetised rats.

Blood pressure and blood gas[2] parameters remained within the normal physiological range during anaesthesia. As expected, blood pressure values were slightly lower than reported values in non-anaesthetised rats (Table 3.1).

Parameter	Min - Max	Mean ± SEM	Non-anaesthetised values (Culley et al., 2003)
Mean arterial blood pressure 1hr after induction of anaesthesia (mmHg) (n=20)	82 - 148	104 ± 4	113 ± 3
Mean arterial blood pressure 2hr after induction of anaesthesia (n=20)	82 - 147	102 ± 4	113 ± 3
pH (n=9)	7.39 – 7.5	7.45 ± 0.01	7.45 ±
pO_2 (mmHg) (n=9)	64 - 186	104 ± 15	84 ± 6
pCO_2 (mmHg) (n=9)	38.2 - 55	43.7 ± 1.8	36 ± 1

Table 3.1: Blood pressure and blood gas parameters during anaesthesia remained within the normal physiological range. Rats received 2 hours of anaesthesia, with 1.2% isoflurane and 70% nitrous oxide, 48 hours prior to spatial novelty preference testing. Blood gas samples were taken at the end of the 2hr period of anaesthesia. Non-anaesthetised values are taken from Culley et. al., 2003 (Culley 2003). Data are presented as mean +/- standard error of the mean (sem).

[2] Due to a technical difficulty, analysis of 11 of the blood gas samples was not possible.

Behavioural testing: spontaneous spatial novelty preference

The amount of time the rat spent in each of the three spatially distinct arms, and the number of entries into each arm, were recorded during both the Exposure and Test phase. Data were compared using repeated measures ANOVA with anaesthesia group (2 levels) as the between-subjects factor and day of testing (3 levels) as the within-subjects factor. Fisher's LSD post-hoc analysis was performed.

Exposure phase

Both anaesthetised and control rats behaved similarly during the Exposure phase and, specifically, there were no group differences in exploration or levels of activity that could account for differences in performance during the subsequent Test phase. There was no effect of anaesthesia on the total number of arm entries (entries made into the Exposure arm plus entries made into the Start arm) made during the Exposure phase (main effect of group ($F[1,38] = 0.784$, $p = 0.38$; mean number of arm entries +/- standard error of the mean (sem) controls: 12 +/- 1.3; anaesthesia: 13.1 +/- 1.2). There was no day by group interaction ($F[2,76] = 0.029$, $p = 0.972$) for total arm entries during the Exposure phase. Total arm entries during the Exposure phase tended to decrease across day but this result did not reach statistical significance (main effect of day: $F[2,76] = 2.61$, $p = 0.08$). There were no main effects of day ($F[2,76] = 0.846$, $p = 0.43$) or group ($F[1, 38] = 0.595$, $p = 0.45$) and no day by group interaction ($F[2,76] = 0.016$, $p = 0.9$) for entries into the Start arm. Entries into the Exposure arm decreased across day (main effect of day $F[2,76] = 5.77$, $p = 0.005$) but there was no main effect of group ($F[1,38] = 0.89$, $p = 0.35$), or a day by group interaction ($F[2,76] = 0.225$, $p = 0.64$).

Test phase

One control rat did not make arm choices in the Test phase and was excluded from that phase. During the Test phase both groups spent the majority of their time exploring the Novel arm (time spent in Novel arm mean +/- sem controls: 361.8 +/- 16 s; anaesthesia: 336.5 +/- 22 s) and made a similar number of arm entries into the Novel arm (mean +/- sem controls: 3.8 +/- 0.18; anaesthesia: 3.6 +/- 0.24). There were no main effects of day ($F(2,74) = 0.287$, $p = 0.75$) or group ($F(1,37) = 0.646$, $p = 0.43$), and no day by group interaction ($F(2,74) = 0.246$, $p = 0.78$) for time spent in the Novel arm. There was a main effect of day ($F(2,74) = 6.936$, $p = 0.002$) for entries into the Novel arm, with entries decreasing across day, but no main effect of group ($F(1,37) = 0.367$, $p = 0.55$) and no day by group interaction ($F(2,74) = 0.405$, $p = 0.67$).

Preference for the Novel arm (time spent and entries made) was calculated as a percentage discrimination ratio (Novel divided by the sum of Novel and Exposure x 100). There was no main effect of day ($F(2,74) = 0.82$, $p = 0.45$) or group ($F(1,37) = 1.53$, $p = 0.22$) and no day by group interaction ($F(2, 74) = 0.26$, $p = 0.77$) for the discrimination ratio of time spent in the arms (Fig 3.3). There was a trend towards a decrease across day for the discrimination ratio of entries into the arms (main effect of day: $F(2,74) = 3.023$, $p = 0.055$) (Fig 3.4). This may suggest that the study was underpowered to detect a difference across day for discrimination ratio. The decrease most likely related to a general increase in familiarity of the Y maze across day (see Discussion). However, there was no main effect of group $F(1,37) = 0.007$, $p = 0.934$, or day by group interaction $F(2,74) = 0.194$, $p = 0.824$. Thus, anaesthesia had no effect

on preference for the Novel arm, suggesting that short-term spatial memory for the Exposure arm was unaffected.

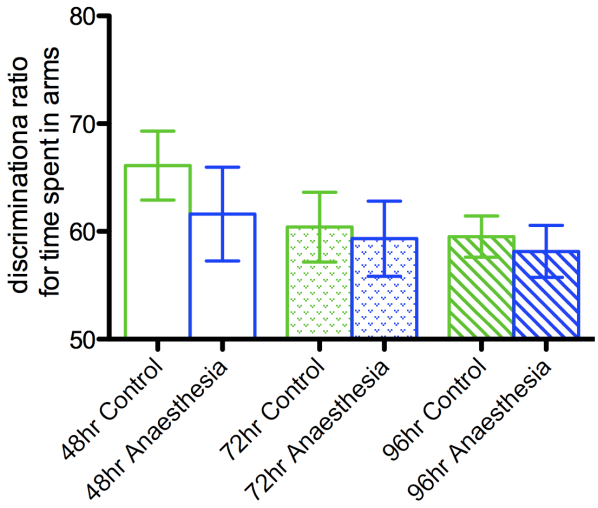

Figure 3.3. Anaesthetised rats spent a similar percentage of time as control rats in the Novel arm, on each of the three post-anaesthetic days. A percentage discrimination ratio [time spent in Novel arm / (time spent in Novel + Exposure arm)] x 100) was calculated for rats that had received anaesthesia (blue bars) and control rats (green bars) at 48 (no fill), 72 (dotted fill) and 96 (hatched fill) hours post-anaesthesia. Data are presented as mean +/- sem 50% time spent in the Novel arm represents chance performance, all rats spent a greater percentage of time in the Novel arm than expected by chance.

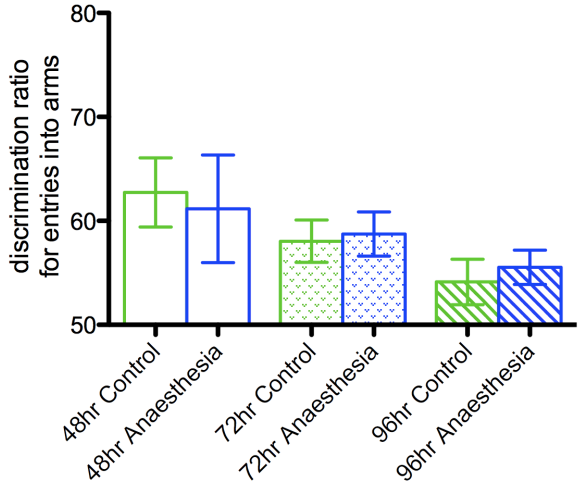

Figure 3.4. Anaesthetised rats made a similar percentage of arm entries as control rats into the Novel arm, on each of the three post-anaesthetic days. A percentage discrimination ratio [entries into Novel arm / (entries into Novel + Exposure arm)] x 100 was calculated for rats that had received anaesthesia (blue bar) and control rats (green bar) at 48 (no fill), 72 (dotted fill) and 96 (hatched fill) hours post-anaesthesia. Data are represented as mean +/- sem 50% entries into the Novel arm represents chance performance, all rats spent a greater percentage of time in the Novel arm than expected by chance.

The strongest novelty preference may be expected to occur upon the first choice of the first trial, as this is when the Exposure arm is the most familiar and the Novel arm is completely novel. The first arm choice (either Novel or Exposure arm) for rats in each group (anaesthesia and control) were compared using Fisher's exact test. There was no difference in first arm choice between rats that received anaesthesia and the control condition ($p = 0.24$); anaesthetised rats made 89.5% of first arm choices into the Novel arm and control rats made 70% of first arm choices into the Novel arm.

Experiment 2: Win-shift on the radial arm maze

Anaesthetic physiology

Body temperature was successfully maintained between 36.5°C and 37.5°C in all anaesthetised rats. Blood pressure and blood gas parameters remained within the normal physiological range during anaesthesia, and were similar to previously reported values in non-anaesthetised rats (Table 3.2). No differences in anaesthetic physiology were found between the two anaesthesia groups (single exposure and multiple exposure), supporting the conclusion that potential group differences in performance on the RAM cannot be explained by differences in anaesthetic physiology. Supporting statistical analyses are presented below.

Blood pressure data collected from Group 1A during anaesthesia, and Group 3A during the third period of anaesthesia, were compared using repeated measures ANOVA with group (2 levels) as the between-subjects factor and sample (2 levels: 1hr and 2hr post-induction) as the within-subjects factor. There were no main effects of group ($F[1,14] = 0.189$, $p = 0.67$) or sample ($F[1,14] = 1.072$, $p = 0.32$), and no group

by sample interaction ($F[1,14] = 0.176$, $p = 0.68$) for blood pressure data from Groups 1A and 3A.

In order to investigate a potential cumulative effect of anaesthesia on blood pressure in the group that received multiple anaesthetics, blood pressure data from Group 3A were compared using repeated measures ANOVA, with phase (3 levels: 1st, 2nd and 3rd anaesthetic) and sample (2 levels) as the within-subjects factors. There were no main effects of phase ($F[2,14] = 1.83$, $p = 0.2$) or sample ($F[1,7] = 1.7$, $p = 0.23$) for blood pressure within Group 3A. Blood pressure within Group 3A was numerically higher for the second samples (after 2 hours of anaesthesia) during the first phase of anaesthesia but the phase by sample interaction was not statistically significant ($F[2,14] = 2.3$, $p = 0.083$).

Blood gas data[3] from Group 3A during the 1st and 2nd anaesthetic phase (exposure) were compared using paired samples t-tests. There were no differences between the 1st and 2nd anaesthetic phase for pH ($p=0.37$), pCO_2 ($p=0.19$) or pO_2 ($p=0.23$).

[3] Due to a technical difficulty, analysis of blood gas samples from Group 1A, and Group 3A during the 3rd anaesthetic phase (as well as one sample from Group 3A during the 2nd anaesthetic phase), was not possible.

Parameter	Min - Max	Mean ± SEM	Non-anaesthetised values (Culley et al., 2003)
Mean arterial blood pressure 1hr after induction of anaesthesia (mmHg) (26 samples)	94 - 141	114 ± 2	113 ± 3
Mean arterial blood pressure 2hr after induction of anaesthesia (26 samples)	96 - 153	118 ± 3	113 ± 3
pH (15 samples)	7.34 – 7.47	7.43 ± 0.01	7.45 ± 0.02
pO_2 (mmHg) (15 samples)	72 - 109	89 ± 3	84 ± 6
pCO_2 (mmHg) (15 samples)	38.7 – 59.3	46.4 ± 1.3	36 ± 1

Table 3.2: Blood pressure and blood gas parameters during anaesthesia remained within the normal physiological range. Rats received either one 2 hr exposure to anaesthesia, with 1.2% isoflurane and 70% nitrous oxide, or three 2 hr exposures to the same anaesthetic regimen, prior to win-shift radial arm maze testing. Blood gas samples were taken at the end of the 2hr period of anaesthesia. Data from both groups and across the phases of anaesthesia for Group 3A are combined here for simplicity, as there were no group or phase differences in intra-anaesthetic physiology. Non-anaesthetised values are taken from Culley et. al., 2003 (Culley 2003). Data are presented as mean +/- standard error of the mean (sem).

These data support the conclusion that any potential group differences in performance on the RAM cannot be explained by differences in physiology during anaesthesia or some cumulative effect of anaesthesia on intra-anaesthesia physiology.

Behaviour testing: win-shift radial arm maze

Total arm entries

All rats made at least 12 choices per trial. The total number of arm entries per trial was recorded along with the time taken to complete the maze. From this the total number of arm entries per minute was calculated, as a measure of maze exploration. This calculation was carried out for the first three trials only, in order to compare the initial exploratory behaviour before, the potential confound of, group separation of performance might have occurred.

During testing, errors were scored when a rat re-entered an arm that it had previously entered during that trial. The number of errors made within the first 12 choices, and the number of choices made before the first error, within a testing trial were recorded. Data were compared using repeated measures ANOVA, with day of testing (9 levels) as the within-subjects factor and anaesthesia group (3 levels) as the between-subjects factor. Fisher's LSD post-hoc analysis was performed.

The total number of arm entries made per minute during a trial was compared across the first three days of testing. There were no main effects of day ($F(2,38) = 1.53$, $p = 0.23$) or group ($F(2,19) = 2.4$, $p = 0.118$), and no day by group interaction ($F(4,38) = 0.635$, $p = 0.64$). In other words group differences in performance cannot be explained by differences in the speed with which the maze arms were explored during the initial phase of testing.

Time to complete the maze

The win-shift performance of all rats improved across testing day. There was a main effect of day of testing for time to complete the RAM ($F[8,152] = 34.903$, $p < 0.0005$), choices before the first error ($F[8,152] = 11.446$, $p < 0.0005$) and errors within the first twelve choices ($F[8,152] = 17.884$, $p < 0.0005$), demonstrating that rats took less time to complete the RAM and made fewer errors across testing days.

There was a main effect of group ($F[2,19] = 7.261$, $p = 0.005$) for time to complete the maze and a group by day interaction ($F[16,152] = 1.739$, $p = 0.045$) (Fig 3.5). Pair- wise comparisons revealed that this effect was predominantly due to a difference between Group 0A and Group 3A ($p = 0.001$) (inset Fig 3.5); demonstrating that control rats were faster to complete the maze than those that

received three episodes of anaesthesia. There was also a similar trend towards a difference between Group 1A and Group 3A ($p = 0.06$) and Group 0A and Group 1A ($p = 0.06$); reflecting that rats that received one episode of anaesthesia tended to be numerically slower to complete the maze than control rats but numerically faster than those that received 3 episodes of anaesthesia. This lack of statistical significance suggests that the study may have been underpowered to detect this difference between groups other than the most extreme comparison (Group 0A and Group 3A).

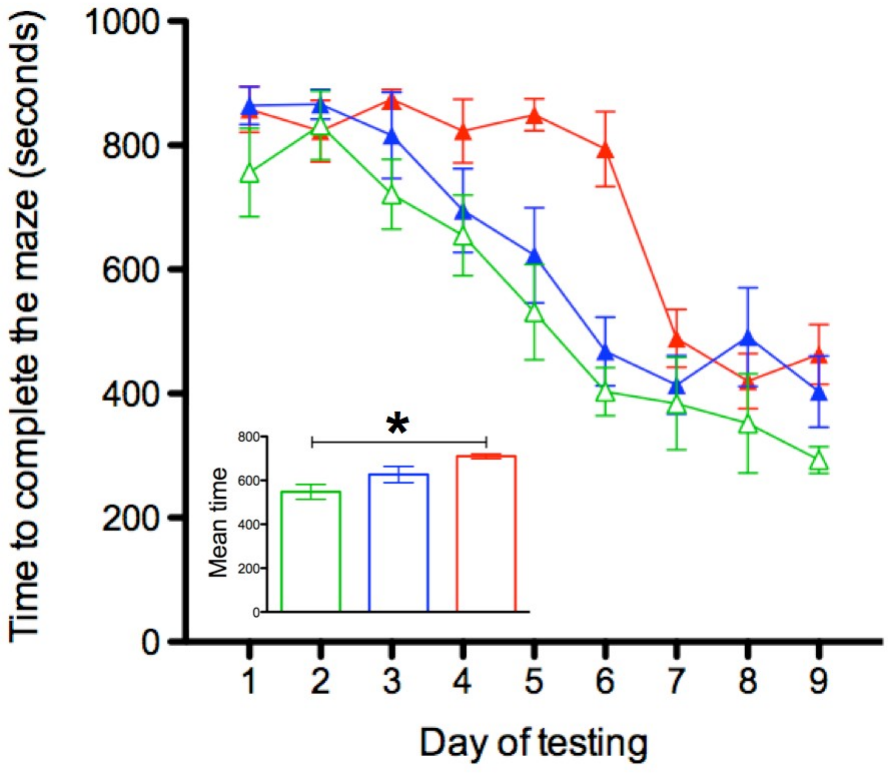

*Figure 3.5. Rats that received repeated episodes of anaesthesia took longer to complete the maze than control rats, or those that received a single episode of anaesthesia. There were main effects of day and group for the time taken to complete the RAM for Group 0A: control rats (green), Group 1A: rats that received one episode of isoflurane and nitrous oxide anaesthesia (blue) and Group 3A: rats that received three episodes of isoflurane and nitrous oxide anaesthesia (red). Fishers LSD post hoc analysis of mean performance across trials (inset) revealed a difference between Group 0A and 3A for the mean time taken to complete the maze. * $p < 0.05$.*

Number of choices before the first error occurred

There were main effects of group for both error measures (Figs 3.6 and 3.7). Group wise comparisons for the number of 'choices before the first error' (main effect of group: $F[2,19] = 5.587$, $p = 0.012$) revealed that there was a difference between Group 0A and Group 1A ($p = 0.035$) and between Group 0A and Group 3A ($p = 0.004$) (inset Fig 3.6); with control rats making more (correct) choices before their first error occurred.

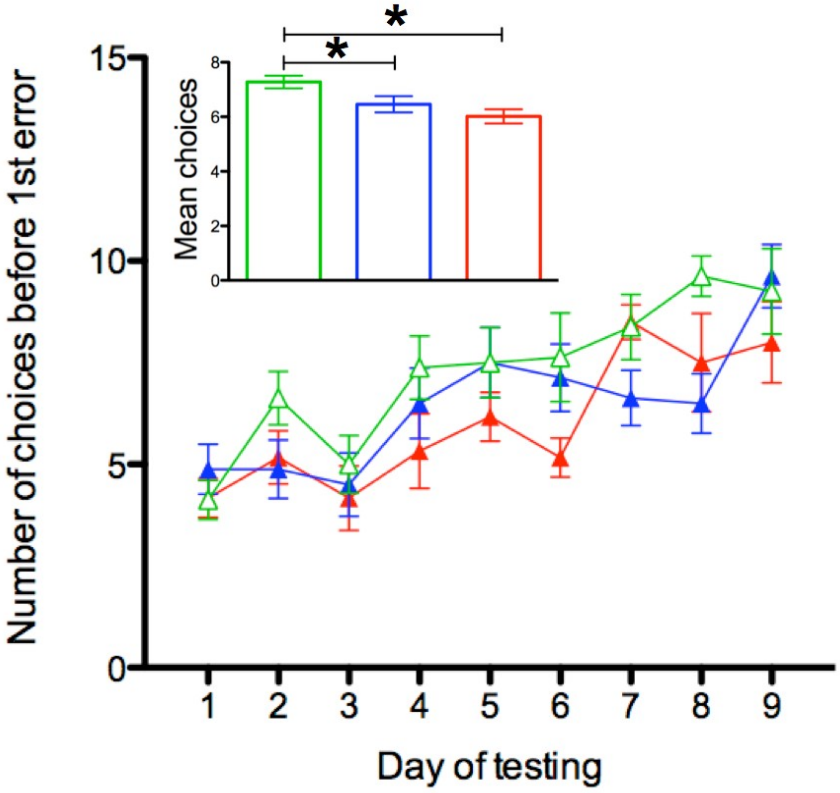

*Figure 3.6. Rats that received anaesthesia made fewer (correct) choices before their first error than control rats, and this effect was dependent on the number of exposures. There were main effects of day and group for the number of choices made before the first error occurred for Group 0A: control rats (green), Group 1A: rats exposed to one episode of isoflurane and nitrous oxide anaesthesia (blue) and Group 3A: rats exposed to three episodes of isoflurane and nitrous oxide anaesthesia (red). Fishers LSD post hoc analysis of mean performance across trials (inset) revealed differences between Group 0A and Group 1A and Group 0A and Group 3A for the total mean number of choices before the first error occurred. *$p < 0.05$.*

Group wise comparisons for 'errors within the first twelve choices' (main effect of group: $F[2,19] = 4.563$, $p = 0.024$), revealed that the effect was predominantly driven by a difference between Group 0A and Group 3A ($p = 0.007$) (inset Fig 3.7), with control rats making fewer errors. In addition, rats that received 1 episode of anaesthesia tended to make fewer errors than those that received 3 episodes, but this effect did not reach statistical significance ($p = 0.082$).

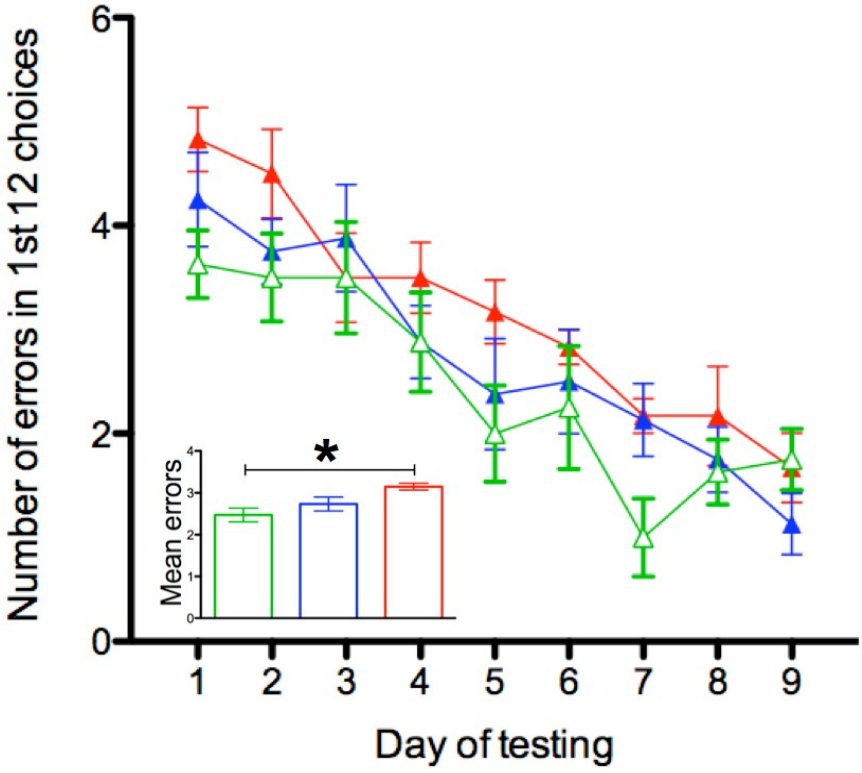

*Figure 3.7. Rats that received repeated episodes of anaesthesia made a greater number of errors in the first twelve choices than control rats, or those that received a single episode. There were main effects of day and group for the number of errors made within the first twelve choices for Group 0A: control rats (green), Group 1A: rats that received one episode of isoflurane and nitrous oxide anaesthesia (blue) and Group 3A: rats that received three episodes of isoflurane and nitrous oxide anaesthesia (red). Fishers LSD post hoc analysis of mean performance across trials (inset) revealed a difference between Group 0A and Group 3A for the total mean number of errors made within the first twelve choices. *$p < 0.05$.*

Experimental conduct

Post-anaesthetic daily food consumption was unaffected by 2 hours of isoflurane and nitrous oxide anaesthesia. Overnight food consumption was recorded, from rats in Experiment 1, for each of the first two days post-anaesthesia. The data were collected in order to investigate whether anaesthesia led to changes in food consumption that could alter the behaviour of rats in Experiment 2, that were on a food restriction protocol.

Overnight food consumption varied between 12 and 26 grams per 24 hours (mean +/- sem controls: 17.57 +/- 1.19; anaesthetised 19.08 +/- 1.13). Data were compared using repeated measures ANOVA with post-anaesthetic day as the within-subjects factor (2 levels) and anaesthesia group as between-subjects factor (2 levels). There were no main effects of day $F[1, 38] = 2.51$, $p = 0.12$, or group $F[1,38] = 0.057$, $p = 0.81$ and no day by group interaction $F[1,38] = 0.46$, $p = 0.5$.

Discussion

Spontaneous spatial novelty preference is not altered by anaesthetic exposure during adulthood, even though the same anaesthetic regimen causes a persistent deficit in win- shift RAM performance. Furthermore, the effect of anaesthesia on RAM performance is dependent on the number of episodes of anaesthesia, despite a seven-day recovery period between successive anaesthetics; demonstrating that repeated exposure to anaesthesia is more detrimental than a single episode.

Spatial novelty preference was not impaired by anaesthesia

Impairment in post-anaesthetic SSNP was not detected, in rats that received one episode of isoflurane plus nitrous oxide anaesthesia. The only difference found was that both control rats and rats that received anaesthesia demonstrated a decrease across day of testing, for entries into the Exposure arm during the Exposure phase, and into the Novel arm during the Test phase. For logistical reasons the order of rooms in which rats were tested, for each of the three post-anaesthetic testing days, was not counterbalanced. It is therefore possible that, by chance, discrimination of the maze arms was harder in the second room compared to the first and again in the third compared to the second. This seems unlikely however, and it is proposed that the most likely explanation for the decrease in discrimination ratios across day merely represents a general increase in familiarity of the Y maze, leading to a progressive decrease in exploration.

Spatial win-shift performance was impaired by anaesthesia

The win-shift RAM performance of rats that received one 2hr period of anaesthesia (Group 1A) was impaired relative to controls (Group 0A); although the effect did not reach statistical significance in all performance measures. The result reproduces that of Culley et al, who found that the same anaesthetic regimen is associated with a persistent deficit in win-shift RAM performance, that is not explained by differences in locomotor ability, as separately assessed by swim speed (Culley et al., 2004b).

Both studies found that previous exposure to isoflurane and nitrous oxide anaesthesia produced a decrease in the number of correct choices made before occurrence of the first error. However, unlike the result here, Culley et al found that adult rats exposed to isoflurane plus nitrous oxide also took longer to complete the maze than control rats (here there was a trend only [$p = 0.06$]). Subjective comparison of control group performance of rats in the Culley study and rats in the study presented here, shows that Culley control rats complete the maze in a slightly faster time throughout. This is not surprising given that there was no inter-choice interval used in the Culley study (personal communication (Culley, 2008)), unlike here where rats were confined to the centre of the maze for five seconds between choices. However, similar comparison of anaesthetised group performance shows that - although Culley rats complete the maze slightly faster initially - during the mid point of testing (days 4-6) Culley anaesthetised rats are in fact slower to complete the maze than anaesthetised rats here (approximately 700 c.f. 590 seconds).

Tne important difference between the two studies is the strain of rat used. The effect in Culley et al was demonstrated using Fischer-344 rats, where as the result here was

found using Lister Hooded rats (a strain akin to the Long-Evans and chosen to represent the strain most commonly used for behavioural neuroscience experiments). Rat strain differences in the performance of the win-shift RAM task (Higashida and Ogawa, 1987, Crusio and Schwegler, 2005), as well as other spatial tasks (Harker and Whishaw, 2002, Crusio and Schwegler, 2005) have been reported. In their study, Higashida & Ogawa investigated strain differences in the performance of rats that were permitted to make successive arm choices (i.e., no inter-choice interval was imposed, thereby enabling the rat to use an egocentric 'chaining' strategy). Interestingly, Higashid & Ogawa found that when rats that used the chaining strategy were treated with a drug that impairs win-shift performance (the muscarinic antagonist scopolamine), they were more impaired than treated rats that did not use the chaining strategy. This raises the possibility that Culley rats, using the chaining strategy, could be more susceptible to drugs that impair win-shift performance. It should be noted that there is inconsistency here, as an earlier study contradicts the result found by Higashida & Ogawa, and suggests that although impairment was dependent on the strategy used, rats using a chaining strategy showed facilitated performance (Watts et al., 1981). Strain differences are not the only difference between this study and that of Culley et. al., that might explain that anaesthetised rats were slower to complete the maze in the Culley study but were not here. Unlike error parameters, 'time to complete the maze' is potentially affected by salience of the spatial cues as well as locomotive ability and motivation to run the maze. As the two studies

were carried out in different laboratory environments and on different apparatus, these factors will have differed. If the salience of spatial cues were greater in the study presented here (despite care to match the salience of cues), this study may have been underpowered to detect a difference in the 'time to complete the maze'

parameter. It should be noted that the number of rats used here was eight per group, rather than five per group as in the Culley study, which would increase statistical power to detect an effect here. However, it is not possible to entirely rule out that effects on motivation or locomotion, as a result of subtle differences in experimental design, could have more impact because both studies used a relatively low number of animals.

Why is performance of the win-shift task but not the SSNP task impaired by anaesthesia

The SSNP and the win-shift RAM tasks are both examples of tasks involving spatial alternation or short term working memory. During the Test phase of the Y maze task, rats demonstrate a spontaneous preference for the spatially distinct, previously unvisited arm. During the RAM task rats are rewarded for alternating arm choice with respect to previous choices, i.e., choosing relatively more novel arms and avoiding relatively more familiar arms. It is not known whether the performance of alternation tasks depends on the active maintenance and manipulation of information (acquired ability akin to working memory in humans) (Olton and Papas, 1979), or rather a passive habituation to previously encountered spatial stimuli (innate ability of rodents, possibly more akin to attentional processing in humans) (Sanderson and Bannerman, 2012). In either case both tasks rely on the use of trial-specific allocentric spatial information and depend on hippocampal and GLUA1 AMPA receptor function (Sanderson and Bannerman, 2012, Sanderson et al., 2007). Phosphorylation of GLUA1 AMPA receptors has been shown to be sufficient to lower the threshold for the induction of long-term potentiation (a long-lasting increase in neuronal signalling)

within the hippocampus, and is therefore thought to lead to synaptic plasticity as a mechanism for enhancement of learning and memory (Makino et al., 2011). The performance of both tasks is adversely affected by deletion of the GLUA1 AMPA receptor subunit though, so this potential mechanism is an unlikely candidate. Why then, does exposure to isoflurane plus nitrous oxide impair the performance of the RAM task but not the Y maze task?

Rats performing the RAM task were food restricted in order to increase motivation to complete the task. Thus, one potential explanation for the deficit in RAM performance is that anaesthesia decreases the effectiveness of food reward, for example by inducing post-anaesthetic nausea. However, there was no difference in daily food consumption between anaesthetised and control rats at 24 and 48hrs post-anaesthesia, the period immediately prior to behavioural testing. In addition, and consistent with the habituation theory proposed by Sanderson and Bannerman, it has been proposed that when rats perform the win-shift RAM task, the novelty of visiting a previously unvisited arm provides motivation for that choice, and food reward is less relevant (Sanderson and Bannerman, 2007, Gaffan and Davies, 1981). It therefore seems unlikely that alteration in type or level of motivation may account for the different findings.

Rats performing the RAM task showed improved performance across trials. This expected improvement has been referred to as a demonstration of rule learning, i.e., learning the win-shift strategy facilitates performance across trials. If rule learning is required for efficient completion of the RAM task, impairment in this process (rather than in the psychological processes involved in spatial discrimination, which are required for both tasks) could explain the poorer performance of rats that received

anaesthesia, and in turn may implicate alternative neurobiological substrates. It has been suggested however, that rather than acquisition of a rule, training-induced improvement in win-shift RAM performance is a reflection of the relative ability in which spatial locations are discriminated, i.e., a perceptual learning effect (Sanderson and Bannerman, 2012, Trobalon et al., 1991). Indeed rodents often perform well above chance at the start of testing on tasks of spatial alternation (e.g., (Bannerman et al., 1999)), strong evidence that rule learning is not necessary for their completion.

When comparing results from the SSNP task and the win-shift RAM task it should be noted that the expected variation in results from healthy animals is different for these tasks. The SSNP task tests spontaneous explorative behaviour, which is inherently more variable. Also of note however, is that the number of animals per group used for the SSNP task experiment was greater (n=20) than for the RAM task experiment (n=8 for the single exposure and control groups). In spite of the increase in statistical power afforded by the increased subject number for the SSNP (Y maze) experiment, anaesthetic induced cognitive impairment was not present. The most likely explanation for the differential effects of anaesthesia on Y maze and RAM performance is that the Y maze task places less demand on memory. Memory load refers to the total amount of cognitive effort being used during working memory.

There is an increased memory load associated with RAM performance because there are twelve rather than two spatial locations for the rat to discriminate between, within a trial, therefore leading to greater sensitivity of the test to detect cognitive impairment. The lack of impairment in Y maze performance is consistent with the hypothesis that the Y maze paradigm is not sufficiently sensitive to detect post-anaesthetic cognitive impairment. It is therefore concluded that short-term spatial

memory deficits after anaesthesia may be related to the memory demand of the particular task used. Furthermore, the lack of impairment on the SSNP task indicates that a primary failure in preference for spatial novelty, or ability to detect novelty of spatial locations, cannot entirely account for the deficit in RAM performance following a single exposure to isoflurane plus nitrous oxide anaesthesia.

Repeat episodes of anaesthesia were more detrimental to win-shift performance than a single episode

Impairment in win-shift RAM performance was detected in both groups that received anaesthesia. The impairment detected in rats that received three exposures to anaesthesia (Group 3A) was more robust than that detected in rats that received one exposure (Group 1A), being of greater magnitude and occurring across multiple performance measures. Importantly there were no differences in physiological parameters, during anaesthesia, between these two groups. Furthermore, animals in both groups made at least 12 choices per trial, there were no group differences in the total number of arm entries per minute made during the early trials, and the mean time to complete the maze was the same for each group on the first day of testing (Fig 5). This suggests that the initial performance of each group was similar and that there were similar levels of exploration. Collectively these results imply that win-shift RAM performance depends on the number of exposures to anaesthesia, and that this cannot be explained by some cumulative effect of anaesthesia leading to a relative hypoxia, hypercapnia or hypotension on successive anaesthetics; or by gross methodological limitations, such as differences in exploration, at least using these measures.

The mechanism(s) by which post-anaesthetic cognitive impairment occurs, and the characteristics of the phenotype itself, may differ depending on the life stage at exposure. That said, the increase in impairment associated with an increased number of exposures, detected here, is congruent with studies in rats and humans demonstrating that repeated exposures to anaesthesia during development (rather than adulthood) are more detrimental to cognitive function than a single exposure (Murphy and Baxter, 2013, Wilder et al., 2009, Sprung et al., 2012). This is consistent with the conclusion that the number of exposures is an important factor affecting the phenotype of post-anaesthetic cognitive impairment in adult rats.

To the author's knowledge, there are no studies that directly compare the post-anaesthetic behaviour of adult rats that have received a single exposure to anaesthesia to those that received repeated exposures. Mice (5-6 months of age) exposed to 1hr of either 1.5 or 2.5% sevoflurane monthly for four months are impaired relative controls when tested on a task of spatial reference memory in the Morris water maze, seven days after the last anaesthetic (Le Freche et al., 2012). Taken together with the study presented here, this suggests that both short term habituation performance on the RAM (otherwise termed working memory) and reference memory performance on the Morris water maze are impaired by prior exposure to anaesthesia; despite the two processes being dependent on different neurobiological mechanisms e.g., different NMDA and AMPA receptor subtypes (Bannerman et al., 2008). There are a number of differences between the two studies though, and future studies could investigate the working and reference memory performance of animals of the same species, that have undergone the same anaesthetic regimen.

An additional study that compared the effects of repeat exposure to those of control condition, found that mice exposed to 2hrs of isoflurane (0.9-1%) or halothane (0.8-1%) daily for five days were not impaired when tested on a standard task of spatial reference memory in the Morris water maze (Bianchi et al., 2008). The results of Bianchi et al appear to be in contrast to either those of Freche et al or the study presented here. However, mice in the Bianchi study received training on the Morris water maze *before* anaesthetic exposure, and so performance depended on retrograde memory (memory for information encountered before anaesthetic exposure) rather than just anterograde memory (memory for information encountered after anaesthetic exposure). Interestingly (in the Bianchi study), when the spatial location of the escape platform within the Morris water maze was moved, at the end of the post-anaesthetic testing phase, wild-type mice previously exposed to isoflurane were impaired relative to control rats. Thus suggesting that when performance is independent of retrograde memory processes, prior exposure to repeat episodes of anaesthesia does indeed impair performance.

Possible mechanisms for anaesthetic-induced impairment in win-shift RAM performance

Both isoflurane and nitrous oxide are known to be neurotoxic when administered to rodents in adulthood. Isoflurane has been shown to increase inflammatory cytokine (interleukin-1-beta) expression and reduce neuronal density, in the hippocampus of rats (Lin and Zuo, 2011). Nitrous oxide exposure is known to cause an increased number of vacuolated neurones in the retrosplenial cortex (Jevtovic-Todorovic et al.,

1998, Jevtovic-Todorovic et al., 2001). Lesions of either the hippocampus (McDonald and White, 1993) or retrosplenial cortex (Vann and Albasser, 2009) are associated with impairment of win-shift RAM performance in rats, and so neurotoxic effects of anaesthesia at these sites provide possible mechanisms for post-anaesthetic impairment task performance. When anaesthesia is administered during development (rather than adulthood), anaesthetic-induced cognitive impairment is strongly associated with neuronal apoptosis (Loftis et al., 2012) and, at least for isoflurane, there is evidence to suggest that the apoptosis is exposure time- and dose-dependent (Wei et al., 2007). In this experiment, the dose and exposure time of anaesthesia were identical for each anaesthetic exposure but the group with the greatest impairment received the greater number of exposures. Cumulative neuronal apoptosis, or sensitization of the neuronal tissue to future damage, are therefore possible mechanisms by which repeated exposure to anaesthesia in adulthood could lead to greater cognitive impairment than that resulting from a single exposure.

It is of note however, that the extremes of age (rather than young healthy adults) appear to be particularly vulnerable to the neurotoxic affects of anaesthesia (Culley et al., 2007, Jevtovic-Todorovic et al., 2013). The very young are vulnerable seemingly because exposure occurs at a time of intense brain development and the very old because there may be an interaction between anaesthetic exposure and age-related cognitive decline or pre-existing pathology. Young healthy adult subjects might be expected to be more resilient to neuronal loss, if they possess more functional reserve for instance (Stern, 2002). It therefore follows that potential mechanisms relating to the function of remaining neurons, such as inflammatory cytokine up regulation (Lin and Zuo, 2011) or effects on neuronal ultrastructure (Kaech et al., 1999) (see Chapter 7) should also be investigated. As with neuronal

apoptosis it is possible that these changes could occur in a cumulative fashion and/or be accompanied by sensitization to future damage.

This study was not designed to establish whether the same level of RAM impairment would be induced by a single period of anaesthesia of identical dose and total exposure time as that received by Group 3A (ie one 6hr exposure). This question could be addressed in future experiments and clearly has implications for the many situations where long and / or multiple anaesthetics are required, such as *in-vivo* behavioural neuroscience studies. For example, studies involving serial imaging require anaesthesia for restraint, and are on the increase as imaging modalities become more widely available. The data quality of some modalities, e.g., magnetic resonance imaging, is increased with increased data acquisition (either by increasing session length or frequency). Investigating how the unintended detrimental effects of anaesthesia may be related to the design of such experiments will ultimately facilitate scientific validity.

Conclusions

Collectively the results of this study suggest that spatial tasks involving few discriminations may be performed normally postoperatively, although tasks that place a greater demand on short-term memory performance are impaired after anaesthesia, even in healthy young adults. It therefore follows that the spontaneous spatial novelty preference task is not a suitable task for investigating anaesthetic-induced cognitive impairment in rats. Further more, these results demonstrate that repeat exposure to anaesthesia is more detrimental than a single exposure. What has yet to be investigated is whether the post-anaesthetic impairment in spatial win-shift performance demonstrated is the result of hippocampal dysfunction *per se* and / or includes a disruption in attentional processing.

CHAPTER 4: EXPOSURE TO ANAESTHESIA DURING ADULTHOOD DOES NOT IMPAIR ASPECTS OF ATTENTION

Introduction

The term 'attention' refers to a heterogeneous construct of psychological processes that enable the prioritisation of relevant information from the environment, in order to inform ongoing behaviour (Sarter et al., 2006b, Robbins, 1997). Varieties of attentional processing include sustained attention: the ability to attend to information over time, selective attention: the ability to attend to a particular subset of information, and divided attention: the ability to attend to more than one piece of information. Successful performance on a behavioural task may involve co-activity of a number of attentional processes. Overall this form of 'top-down processing' may be referred to as executive function or cognitive control and is an essential component of cognitive processes such as working memory, problem solving and task flexibility.

Cognitive impairments may occur after general anaesthesia including post-operative cognitive dysfunction (POCD). To date no literature exists on the presence or absence of attention deficits in animal models of POCD; however, data from some human patient studies suggest that such impairments may occur (Moller et al., 1998, Price et al., 2008). The first multi-centre study of POCD was that of the International Study of Post-operative Cognitive Dysfunction group (ISPOCD), published in 1998. Approximately 1200 patients were recruited from 14 European Institutions having presented for non-cardiac thoracic, major abdominal or orthopaedic surgery with

general anaesthesia. They undertook a battery of neuropsychological tests before, 1 week after and 3 months after surgery and anaesthesia. Within this patient population the group found an incidence of POCD at 1 week post-operatively of 25.8%, as compared to 3.4% for the control group (Moller et al., 1998). A follow up study of the same data set revealed that the most common deterioration of post-operative cognitive function was seen at 1 week post-operatively in tests that are thought to reflect attention and cognitive speed, as opposed to memory function *per se* (Silverstein et al., 2007). A separate follow up study of the same data set showed that post-operative delirium, a condition defined by acute changes in attentional processing, was a risk factor for the development of early POCD (Rudolph et al., 2008). The patient population for the ISPOCD studies was comprised of patients 60 years old and older, all undergoing surgery and some with co-morbidities, and may not therefore be of relevance to a population of healthy adult laboratory animals. It is of note, none-the- less, that in this patient population impairments in attentional processing appear to be a key feature of the cognitive dysfunction following anaesthesia and surgery.

Data from in-vivo animal experiments regarding the presence of POCD following anaesthesia are compelling. In Chapter 3 here, I have shown that two hours of isoflurane plus nitrous oxide anaesthesia is sufficient to impair the performance of rats in a radial arm maze, spatial working memory task; and that repeated episodes of anaesthesia are more detrimental than a single exposure. One possible cause of poor task performance in this model is impairment in attentional processing. Performance of the spatial working memory radial arm maze task involves choosing to visit previously unvisited arms of the maze. It has been proposed that this ability to alternate arm choice according to previous choices is an innate ability of rodents, and

may be more akin to attentional processing than the acquired ability of working memory (Sanderson and Bannerman, 2012). Sanderson and Bannerman propose that impairment in spatial working memory on the radial arm maze may represent deficits in short term habituation, thereby impairing the ability to habituate to (and therefore recognise as previously visited) familiar arms. In addition, it is certainly the case that in order to complete the task efficiently rats must attend to and visually scan extra maze spatial cues. Impairment in visual attentional processing could therefore impact on the performance of the radial arm maze task by reducing the animals' ability to alternate arm choice and / or attend to the relevant information that is required for maze navigation. Whether anaesthesia would produce attentional impairments was investigated with the use of two behavioural tasks that are commonly used for assessing attention in rats; in order to extend the phenotypic characterisaiton of cognitive deficits after general anaesthesia.

The sustained attention task (SAT) as validated by McGaughy and Sarter (McGaughy and Sarter, 1995) is an operant task that assesses vigilance (a state of readiness to detect and respond to information). Rats are rewarded with food for distinguishing between the presentation and absence of presentation of a visual stimulus (illumination) by choosing a particular one of two levers. The task has been used to characterise the neurobiology of sustained attention in health and disease (Sarter et al., 2003, Turchi and Sarter, 2001, McGaughy et al., 1996, Sarter et al., 2006a), as well as to characterise the effects of drugs (Bushnell et al., 1997) and toxic chemicals (Bushnell et al., 2002, Samsam et al., 2005).

The second task chosen for the investigation of post-anaesthetic attentional impairment was the 5-Choice Serial Reaction Time Task (5CSRTT). The task (as

described by Bari et al (Bari et al., 2008)) requires that rats visually scan and attend to five response windows, present as a horizontal array along the wall of an operant testing chamber. A visual stimulus (illumination) is presented at one of the five ports (a different port on each trial) and the rat is required to nose poke that port in order to receive a food reward. Rather than requiring that rats distinguish between the presentation and absence of presentation of a centrally located visual stimulus (as in the SAT), this task requires attention in both the temporal *and* spatial domains. Since its original development, as the 9-choice task in 1983 (Carli et al., 1983), the 5CSRTT has been used to characterise the neurobiology of sustained and selective attention, as well as that of inhibitory control (as indexed by the number of premature or perseverative responses the rat makes at the response windows) (Robbins, 2002).

In two separate experiments (with two separate cohorts of rats), pre and post-anaesthetic attentional processing in rats performing both the sustained attention task (SAT) and the 5-choice serial reaction time task (5CSRTT) were investigated. The effect of two different anaesthetic regimens (the volatile anaesthetic regimen: isoflurane plus nitrous oxide [as used in Chapter 3 here] and the injectable anaesthetic regimen: propofol plus fentanyl [a commonly used anaesthetic combination]) was investigated in these experiments, along with the relative effect of single versus repeat administration of isoflurane plus nitrous oxide anaesthesia. It was hypothesised that exposure to anaesthesia would lead to post-anaesthetic impairments in attention, and that the impairments would be greater in rats that had been repeatedly exposed to anaesthesia.

Materials and Methods

Subjects

Male Long-Evans rats (Harlan, USA: Experiment 1; Taconic, USA: Experiment 2), 3 months old at the time of training were pair housed, in polycarbonate cages with automatically regulated lighting (12/12h light/dark cycle, lights on at 0700h [Experiment 1] or lights on at 2000h [Experiment 2]). Experiments 1 and 2 were carried out in different animal units due to the location of behavioural testing equipment. This limitation required that the two experiments were carried out in different phases of the light / dark cycle; Experiment 1 was carried out in the light phase and Experiment 2 was carried out in the dark phase.

Experimental design

Experiment 1: Sustained attention task (see Table 4.1 for chronology of experimental phases)

Rats were food restricted from two weeks before training began (see Section 3.3, Chapter 2 - General Methods for details) and underwent training on the task to a pre-determined criterion (see Section 5.3, Chapter 2 - General Methods for details). Prior to baseline testing, rats were allocated to one of three groups for Experiment 1a: control condition (n=9) (Group C); one 2hr period of anaesthesia with isoflurane and nitrous oxide (n=8) (Group i/nA); one, two-hour period of anaesthesia with propofol and fentanyl (n=9) (Group p/fA). Groups were counterbalanced for level of performance (relative number of hits [hits / hits + misses] and the relative number of correct rejections [correct rejections / correct rejections + false alarms]), based on data collected just prior to the final baseline test day, and as far as possible for body weight. Pre-anaesthetic (baseline) testing consisted of one baseline session, 24 hours before

anaesthesia. Post-anaesthetic testing was begun 24 hours after anaesthesia and consisted of one session, of 162 trials, per day for six days, conducted between 1100h and 1400h.

On completion of post-anaesthetic testing and in order to provide a positive control, in Experiment 1b all rats from Experiment 1a received systemic scopolamine - a muscarinic antagonist known to impair attentional processing (McGaughy et al., 1994). Rats received vehicle, low (0.1 mg/kg) and high (0.25 mg/kg) doses of scopolamine subcutaneously, 30 minutes before behavioural testing. Each rat received each condition on separate testing days. Each dose-testing day was separated by three weeks. The order in which rats received the doses was counterbalanced for prior anaesthetic exposure and a within-subjects, latin square design was used. Post-dose testing consisted of one session of the same task version as used in Experiment 1a.

Order	Experimental design phase	Comments
1	Food restriction	From 2 weeks before training began
2	Task training	To criterion of >75% hits to 500ms signals and >75% correct rejections to non-signal trials
3	Group allocation	Counterbalanced by signal detection and correct rejection performance, and as far as possible by body weight
4	Baseline testing	1 day of testing, 24hrs before anaesthesia
5	Anaesthesia	3 groups: Control condition, isoflurane plus nitrous oxide or propofol plus fentanyl
6	Post anaesthesia testing	6 days of testing, from 24hrs after anaesthesia
7	Low dose scopolamine, high dose scopolamine or vehicle administration	Latin square design
8	Post scopolamine testing	6 days of testing, from 24hrs after scopolamine
9	Low dose scopolamine, high dose scopolamine or vehicle administration	3 weeks after previous dose
10	Post scopolamine testing	6 days of testing, from 24hrs after scopolamine
11	Low dose scopolamine, high dose scopolamine or vehicle administration	3 weeks after previous dose
12	Post scopolamine testing	6 days of testing, from 24hrs after scopolamine

Table 4.1 Chronology of experimental phases for Experiment 1 - effect of anaesthesia on performance of the sustained attention task.

Experiment 2: 5-choice serial reaction time task (see Table 4.2 for chronology of experimental phases)

Rats were food restricted from two weeks before training began (see Section 3.3, Chapter 2 - General Methods for details) and underwent training on the task to a pre-determined criterion before pre-anaesthetic (baseline) testing began. After reaching criterion, and prior to baseline testing, rats were allocated to one of two groups for Experiment 2a: control condition (n=8) (Group C), or anaesthesia condition, i.e., 2 hrs

of isoflurane plus nitrous oxide anaesthesia (n=9) (Group i/nA). Groups were counterbalanced for level of performance (percentage of accurate choices and percentage of omissions), and as far as possible for body weight. Pre-anaesthetic (baseline) testing consisted of one session, of 150 trials, per day for six days. Anaesthesia (or control condition) was carried out 24hrs after baseline testing and 24hrs before post-anaesthetic behavioural testing began. Post-anaesthetic testing consisted of one session per day for six days, conducted between 1100h and 1400h.

On completion of post-anaesthetic testing, control rats from Experiment 2a (n = 6) (renamed Group p/fA) were used to investigate the effect of a single 2hr exposure to propofol plus fentanyl on 5CSRTT performance, using a within-subjects design (before and after administration of anaesthesia). In this experiment (Experiment 2b) pre and post-anaesthesia behavioural testing was identical to that in Experiment 2a.

In order to provide a positive control for the 5CSRTT, rats from Experiment 2a and 2b received cholinergic basal forebrain lesions - a manipulation known to impair performance of the 5-CSRTT (McGaughy et al., 2002) - or sham surgery. Rats (lesion n=7; sham n=5) were counterbalanced for prior anaesthetic exposure and as far as possible for body weight. In this experiment (Experiment 2c) post-surgical behavioural testing was carried out following a 10-day recovery period.

Order	Experimental design phase	Comments
1	Food restriction	From 2 weeks before training began
2	Task training	To criterion of >75 correct trials, >75% accuracy and <20% omissions
3	Group allocation	Counterbalanced by signal detection and correct rejection performance, and as far as possible by body weight
4	Baseline testing	6 days of testing, up to 24hrs before anaesthesia
5	Experiment 2a: Isoflurane plus nitrous oxide anaesthesia	2 groups: Control condition or isoflurane plus nitrous oxide
6	Post anaesthesia testing	6 days of testing, from 24hrs after anaesthesia
7	Repeat anaesthesia	
8	Post anaesthesia testing	6 days of testing, from 24hrs after anaesthesia
9	Repeat anaesthesia	
10	Post anaesthesia testing	6 days of testing, from 24hrs after anaesthesia
11	Experiment 2b: Propofol plus fentanyl administered to control rats	
12	Post-propofol plus fentanyl testing	6 days of testing, from 24hrs after propofol plus fentanyl
13	Experiment 2c: Basal forebrain cholinergic lesion surgery	2 groups: lesion or sham (counterbalanced for previous anaesthetic exposure, performance and as far as possible for body weight)
14	Post-surgical testing	6 days of testing, from 10 days after surgery

Table 4.2 Chronology of experimental phases for Experiment 2 - effect of anaesthesia on performance of the 5-choice serial reaction time task.

Anaesthesia and control condition

Isoflurane plus nitrous oxide

Episodes of anaesthesia consisted of 2 hours of 1.2% isoflurane plus a carrier gas combination of 70% nitrous oxide, 30% oxygen (see Section 4, Chapter 2 - General Methods for detailed methods). Indirect blood pressure was recorded after one and

two hours of anaesthesia (i.e. two samples per animal per anaesthetic exposure). In addition, at the end of the anaesthetic episode, a venous blood sample was taken and immediately analysed for pH, pCO_2 and pO_2. The control condition consisted of 2 hours of medical air plus oxygen (fraction of inspired oxygen 0.3), in an identical chamber with identical gas flow rate.

Propofol plus fentanyl

Dose rates for propofol (Diprivan, Fresenius Kabi, USA) and fentanyl (Fentanyl-Janssen, Janssen Pharmaceutica, USA) were chosen, based on experience, to most closely match the depth and physiological condition achieved with the isoflurane and nitrous oxide regimen used in this study. Anaesthesia was induced with 3% isoflurane carried in 100% oxygen in a temperature-controlled chamber (the same equipment as used for the isoflurane and nitrous oxide anaesthetic regimen), until loss of righting reflex. Once removed from the chamber the rat was placed in ventral recumbency with a face mask supplying the anaesthetic gas (2% isoflurane carried in 100% oxygen). Intravenous catheters (24 gauge Angiocath, BD, Oxford UK) were placed in the right and left lateral tail veins. A bolus of propofol (2-6 mg/kg) was administered intravenously to the desired effect (a decrease in respiratory and pulse rate, plus loss of response to toe pinch). Isoflurane administration (approximately 10 mins) was then ceased. Response to toe pinch was judged as withdrawal of the limb, muscular twitching or increase in muscle tone, and/or changes to the rate, depth or pattern of respiration (i.e. motor or autonomic nervous system responses). The two-hour anaesthetic exposure began upon cessation of isoflurane, and anaesthesia was maintained with continuous infusions of propofol (0.6mg/kg/min) and fentanyl (15μg/kg/hr) (Pump 11 plus, Harvard, USA and Lectrocath extension tube, Vygon,

UK). Oxygen (50%) was administered via a facemask for the duration of the procedure; in order to ensure that blood oxygen saturation (pulse oximeter reading) levels matched those for the group anaesthetised with isoflurane and nitrous oxide (i.e. they remained above 95%). Subject monitoring was identical to that for isoflurane and nitrous oxide anaesthesia, including blood gas analysis (see Section 4.2, Chapter 2 - General Methods for details). At the end of the two-hour period, administration of propofol and fentanyl was ceased. Rectal temperature and pulse oximetry were monitored and oxygen administered until return of purposeful movement, at which point the rat was returned to its home cage and observed to ensure a full recovery from anaesthesia (until fully mobile, and eating and drinking).

Control condition

The control condition consisted of 2 hours of medical air plus oxygen (fraction of inspired oxygen 0.3), in an identical chamber to those in which rats were anaesthetised with isoflurane and nitrous oxide, with identical gas flow rate.

Behavioural testing

Experiment 1: Sustained attention task

Apparatus

Animals were trained in sound attenuated operant chambers (Med Associates, VT, USA) equipped with two retractable levers, a house light (2.8 W), a 45 mg pellet dispenser, a 2900 Hz sonalert tone generator, and three panel lights (2.8 W). The food dispenser, panel lights, and retractable levers were all located on the same wall. The levers were located either side of the central food dispenser. The tone generator and

house light were located on the opposite wall. Records of signal presentation, lever operation, and food pellet delivery were maintained using Med-PC software (MedAssociates, VT, USA). Rats were assigned to a particular testing box for the duration of behavioural training and testing.

Lever training

All rats were food restricted before SAT training began and were maintained at 85% of ad libitum levels (see Section 3.3 for details). Training occurred between 11am and 2pm five days per week, and consisted of one 30-minute session per day. Rats were trained to lever press for food, using a fixed-ratio-one schedule (receiving a food reward for each lever press) until they produced at least 50 responses on each of the two levers, for two consecutive days, at which point task training began.

Initially food was placed on the levers to encourage the rats to interact with them. Once rats started to lever press they were rewarded for each press, regardless of which lever was pressed. There was no limit on the number of times a rat could lever press during the 30-minute training session. During this period of lever press training a one-off correction schedule was used if rats developed a side bias (preference for a particular lever). A side bias was defined as pressing either the right or the left lever for more than 70% of the total presses within a session, for more than two consecutive sessions). The correction schedule was a 30-minute schedule (which replaced the following day's session) during which rats were rewarded for pressing their least preferred lever and not rewarded for pressing their preferred lever.

Task training

Rats were food rewarded for distinguishing between signal (central panel and left panel illumination for 1 second) and non-signal (absence of visual stimulus) trials by pressing the appropriate one of two levers extended 2 seconds after presentation (or lack of presentation) of the stimulus. Rats were required to press the left lever on signal trials and the right lever on non-signal trials. The levers remained in place until pressing occurred, for up to a maximum of 4 seconds. The left and right lever function was not counterbalanced, i.e., all rats, in all trials, were required to press the left lever on signal trials and the right lever on non-signal trials.

Correct lever presses were defined as *hits* when they occurred, at the left lever, on a signal trial and *correct rejections* when they occurred, at the right lever, on a non-signal trial. Incorrect lever presses were defined as *misses* when they occurred on a signal trial and *false alarms* when they occurred on a non-signal trial. If the animal failed to respond or responded incorrectly, the levers were retracted, the inter-trial interval (ITI) (12 +/- 3s) was reinstated and the trial was repeated up to three times (correction trials). If the animal failed to respond or responded incorrectly after three correction trials a forced-choice trial occurred. During a forced-choice trial the event (signal or non-signal) was repeated but only the correct lever was extended and remained active for up to 90 seconds (with illumination of the central and left panel if on a signal trial).

These forced-choice trials served to prevent development of a side bias during this phase of training.

Shaping

Once rats responded correctly to at least 70% of signal and non-signal events for at least three consecutive days, the signal was changed to one second illumination of the central panel light only (rather than central plus left panel). Once rats responded correctly to at least 75% of signal and non-signal events for at least three consecutive days, rats began training to criterion with variable signal length.

The length of the signal was modified to 500, 50 or 25 milliseconds. Rats performed for a maximum of 162 consecutive trials or 40 minutes (one session) per day. Each session was divided into three blocks of 54 trials, each counterbalanced for signal length, such that each block consisted of 9 trials of each signal length and 27 non-signal trials. Rats were trained to a criterion of >75% hits to 500ms signals and >75% correct rejections to non-signal trials, for at least two consecutive sessions. Correction trials and forced-choice trials were not used during this phase. Once all rats reached criterion, baseline performance was assessed on the day preceding anaesthesia.

Testing procedure

Post-anaesthetic testing consisted of one session per day for six days. Rats performed for a maximum of 162 consecutive trials or 40 minutes per day. There was no difference between these sessions and the final shaping schedule. Signal duration varied (as in the final shaping schedule), and all trials were presented in a temporally unpredictable fashion to heighten attentional demands.

During each session the number of hits (correct signal-trial lever presses), misses (incorrect signal-trial lever presses), correct rejections (correct non-signal-trial lever

presses), false alarms (incorrect non-signal-trial lever presses) and errors of omission (lack of lever press) were recorded.

Once rats reached criterion, they were allocated to anaesthetic groups, counter-balanced for sex (Chapter 6 only), level of performance (relative number of hits [hits / hits + misses] and the relative number of correct rejections [correct rejections / correct rejections + false alarms]), and as far as possible for body weight. Rats then underwent one day of baseline testing before being anaesthetised.

Behavioural performance measures recorded included signal detection (a percent correct measure: number of hits / number of hits plus number of misses multiplied by 100), correct rejections (a percent correct measure: number of correct rejections / number of correct rejections plus number of false alarms multiplied by 100) and the number of omissions for signal and non-signal trials separately.

Experiment 2: 5-choice serial reaction time task

Apparatus

Rats were trained in Bussey-Saskida Rat Touch Screen Chambers (Model 80604, Lafayette Instruments, IN, USA) using ABET II Software for Touch Screen (Model 89505). Sound-attenuating operant boxes were fitted with a trapezoidal enclosure 33 cm long, 31 cm wide and 30 cm high, covered with a clear acrylic sheet during testing. The large end of the trapezoidal enclosure was comprised of a touchscreen, covered by a black acrylic matt with five 3 x 3 cm response windows cut out in a horizontal plane and at a height just above the rats eye level. The opposite end of the enclosure contained a food magazine into which food pellets were dropped from an automated

dispenser. The magazine was equipped with a light and an infrared beam-break. The chamber also contained a house light and a small speaker. Each rat was assigned to a particular testing box for the duration of behavioural training and testing.

Nose poke training

For a detailed description of the task see (Bari et al., 2008). All rats were food restricted before 5CSRTT training began and were maintained at 85% of ad libitum levels (see Section 3.3, Chapter 2 - General Methods for details). Training occurred between 1100h and 1400h five days per week. Rats were trained to make nose-pokes in any one of five reward windows, using a fixed-ratio-one (FR1) schedule (receiving a food reward for each nose poke). Initially food was placed in each of the response windows to encourage rats to interact with them. Once rats started to nose poke they were rewarded for each poke, regardless of which window was poked. During this phase of nose poke training a one-off correction schedule was used if rats failed to poke a particular window(s). The correction schedule was an additional daily session where only nose pokes at particular windows were rewarded. Once rats were reliably poking each of the 5 windows (decided subjectively by the experimenter via video camera) task training began.

Task training

Rats were required to sustain visual spatial attention (visual scanning) of the five response windows. A brief visual stimulus (illumination) occurred in a pseudo-random location (counterbalanced within a session to ensure equal numbers of illuminations at each of the five response windows), and the rat was required to nose poke that location within a limited time (limited hold: LH). Correct trials were

rewarded with a pellet delivered to the food magazine; a nose-poke in the food magazine was required to initiate new trials, and all trials were separated by an inter-trial interval (ITI), which was 5 seconds long during training and began after the nose-poke in the food magazine. Incorrect responses were punished with a 30s time-out period with the house light on. Trials in which the animals made premature responses or failed to make a response were similarly punished. Initially training sessions consisted of 100 trials in up to 30 minutes. The stimulus duration (illumination) was 30 seconds and the LH (the time allowed between stimulus presentation and nose poke) was 30 seconds. Once the rat reached a pre-determined criterion, the training session parameters were altered to decrease the stimulus duration and LH, and thereby increase the attentional demands. For a list of the training session stages and criterion required see Table 4.3.

Training stage	**Stimulus duration (s)**	**Inter trial interval (ITI) (s)**	**Limited hold (LH) (s)**	**Criterion to move to next stage**
1	30	5	30	≥ 30 correct trials
2	20	5	20	≥ 30 correct trials
3	10	5	10	≥ 50 correct trials
4	5	5	5	≥ 50 correct trials > 80% correct
5	2.5	5	5	≥ 50 correct trials > 80% correct < 20 % omissions
6	1.25	5	5	≥ 50 correct trials > 80% correct < 20 % omissions
7	1	5	5	≥ 50 correct trials > 80% correct < 20 % omissions
8	0.9	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
9	0.8	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
10	0.7	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
11	0.6	5	5	≥ 50 correct trials > 75% correct < 20 % omissions
12	0.5	5	5	≥ 75 correct trials (of 150 total) > 75% correct < 20 % omissions
13	0.5	5 for first 50 trials 2 for middle 50 trials 5 for last 50 trials	5	≥ 75 correct trials (of 150 total) > 75% correct < 20 % omissions

Table 4.3 Overview of steps involved in the training of rats on the 5-choice serial reaction time task. Following nose poke training, training proceeds via 13 distinct criterion-based stages. Stages 1-11 comprise 100 trials in 30 minutes and stages 12 and 13 comprise 150 trials in 45 minutes. Table adapted from (Bari et al., 2008).

Rats were trained to criterion, for five consecutive days, on training stage 13 (>75% accuracy, <20% omissions, maximum 150 trials or 45 minutes of testing) for a maximum of 150 consecutive trials or 45 minutes. This final version of the task included the first and last 50 trials having an ITI of 5s, and the middle 50 trials having an ITI of 2s. The purpose of this increased event rate (number of trials per minute) for the middle 50 trials was to increase the attentional demands of the task (see Fig 4.1 for a schematic representation of the task). After task training was complete rats were allocated to anaesthetic groups, counter-balanced for level of performance (percentage accuracy: correct choices / correct plus incorrect choices, multiplied by 100) and as far as possible for body weight, and pre-anaesthetic testing began. Pre-anaesthetic (baseline) testing consisted of six days of testing, the last of which occurred 24hrs before anaesthesia or control condition. Post-anaesthesia testing occurred 24hrs after anaesthesia and consisted of six days of testing.

Behavioural measures included choice accuracy (a percent correct measure: number of correct responses / correct responses plus incorrect responses, multiplied by 100), omissions (a percent omission measure: number of omitted trials / total number of trials, multiplied by 100), number of premature responses (nose pokes that precede stimulus), number of perserverative responses (repeated nose pokes at the correct response window after a stimulus), the response latency (time taken to respond to a stimulus) and the reward collection latency (time taken to collect food reward).

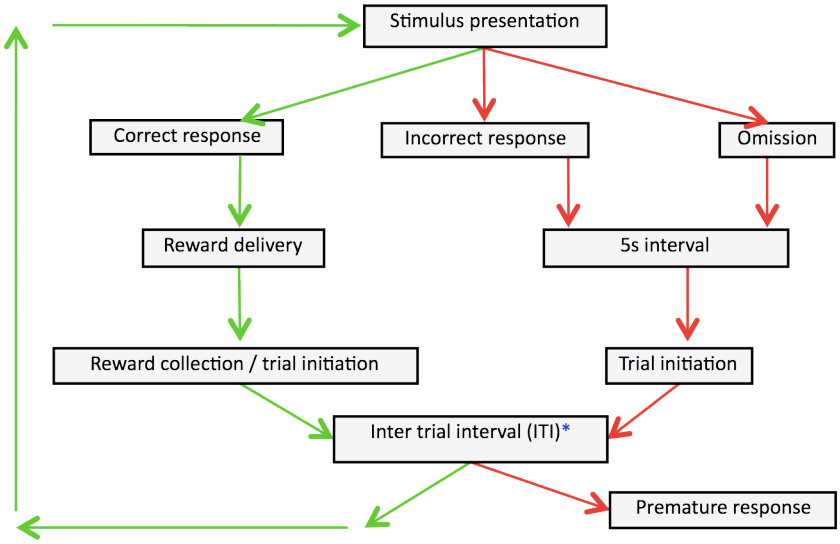

Fig 4.1 Schematic of the 5-choice serial reaction time task. Rats performed 150 consecutive trials (1 session) per day. During a trial, one of five ports was illuminated (each port being illuminated 30 times during a session) and a correct response recorded if the rat nose poked that port within 5 seconds. The event rate - number of trials per minute - was increased for the middle block of trials (*), in order to increase attentional demands of the task.

Surgical procedure for Experiment 2c

Surgical preparation

Surgical anaesthesia was induced with 4% isoflurane carried in 100% oxygen in a temperature controlled induction chamber. Rats were temporarily removed from the chamber for administration of buprenorphine analgesia (Vetergesic: Alstoe, UK), meloxicam analgesia (Metacam: Boehringer Ingelheim, UK) (2mg/kg subcutaneously), bupivacaine local anaesthetic (Marcain: Astra Zeneca, UK) (2mg/kg intradermally at incision site) and saline 0.9% (2ml subcutaneously), and shaving of the surgical site. Rats were then transferred to a nose cone mounted on the stereotaxic frame and maintained on isoflurane (1.5-3.5% as needed). Monitoring consisted of subject pulse oximetry (VitalStore, Vetronic Services, UK), respiratory rate and depth, indirect blood pressure and rectal temperature (Powerlab, ADInstruments, UK) which was maintained between 37°C and 37.5°C. Surgical

preparation consisted of skin cleaning with povidone iodine (Betadine: Sanofi, USA) and alcohol. Protective ointment (Lacrilube: Allergan, USA) was placed in the eyes to prevent dehydration of the corneas.

Surgical technique

The skin over the skull was incised, the skull levelled between bregma and lambda, and small burr holes drilled over the sites into which a 28G flat Hamilton needle would be introduced (two injection sites per hemisphere targeting the basal forebrain: AP – 0.75, ML ± 2.3, DV -7.8; AP – 0.75, ML ± 3.3, DV -8.1 relative to bregma, i.e., a skull surface landmark). The needle was lowered to the appropriate DV coordinate and 0.2 ul 192 IgG-saporin (0.15 mg/ml) (Advanced Targeting Systems, IT-001-25 – 0.15mg/ml) (lesion group) or 0.2 ul phosphate buffered saline (sham group) was infused at 0.1 ul/min, following which the needle was left in place for 3 minutes. The wound was sutured with absorbable sutures and saline 0.9% (2ml subcutaneously) was administered. 192 IgG-saporin is a neurotoxin that targets cells expressing the low-affinity nerve growth factor (p75) receptor on their cell surface (Wiley et al., 1991). Within the basal forebrain, the p75 receptor is only expressed on cholinergic neurons. In this way the cholinergic neurons at a particular location within the basal forebrain can be specifically eliminated, while sparing neighbouring noncholinergic neurons. Performance of the SAT is dependent on the cholinergic neurons of the basal forebrain, and therefore lesioning of those neurones disrupts task performance (McGaughy et al., 1996).

Recovery from surgery

Rats were placed in a warmed cage with 100% oxygen until they were able to move around the cage and respond appropriately to gentle stimulation, at which point oxygen was ceased and they were offered soft palatable food and water. Rats were monitored continuously until eating, drinking and fully mobile. They remained in a recovery cage with a heat mat placed underneath half of the cage floor until the following morning, at which point they were re-paired with their cage mate and returned to the home cage. Meloxicam (2mg/kg per os) was administered 12, 24, 36 and 48 hours post-operatively for analgesia. Rats were allowed to recover for 10 days before post-operative testing began.

Immunohistochemistry

Once behavioural testing was complete, rats from Experiment 2c were anaesthetised with intra-peritoneal pentobarbitone (100mg/ml) and transcardially perfused, at a rate of 25ml/min, with ice cold 1% paraformaldehyde in 0.1 molar phosphate-buffered saline (PBS) (pH 7.4) for three minutes; followed by fixation with 4% paraformaldehyde in 0.1 molar PBS (pH 7.4) for 15 minutes at a rate of 25ml/min.

The brains were carefully dissected and postfixed overnight at 4 °C in 4% paraformaldehyde in PBS, before being cut into 50 micrometer coronal sections on a Vibratome (Leica VT1000S, USA) and stored at 4 °C in PBS. Sections were processed for immunohistochemistry using primary antibodies against choline acetyltransferase (ChAT) (goat monoclonal anti-ChAT; Chemicon AB144P USA) and a rabbit anti-goat secondary antibody (Jackson, USA). Standard avidin-biotin complex with horseradish peroxidase / diaminobenzidine visualization as described

previously (Berger-Sweeney 2000). The reduction of ChAT-immunoreactive neurons confirmed the location of the lesion within the basal forebrain.

Statistical analysis

Experiment 1: Sustained attention task

Blood pressure data collected from groups i/nA and p/fA were compared using repeated measures ANOVA with sample (2 levels: 1hr and 2hr post-induction) as the within subject factor and group (2 levels) as the between-subjects factor. Blood gas data from the same groups were compared using independent samples t-tests.

Behavioural data were compared using repeated measures ANOVA, with day of testing (post-anaesthesia testing only) (6 levels: testing days 1-6), block (3 levels: first, middle and last block of 54 trials) and signal length (signal trial parameters only) (3 levels: 500, 100 and 25 ms) as the within-subjects factors and anaesthesia group (3 levels) as the between-subjects factor. As sample sizes were slightly different, Gabriel's post hoc analysis was performed. Baseline data were analysed separately to post-anaesthesia data.

Experiment 2: 5-choice serial reaction time task

Blood pressure and blood gas data collected from group i/nA during the first, second and third periods of anaesthesia were compared using repeated measures ANOVA, with sample (blood pressure data only) (2 levels: 1hr post-induction and 2hr post-induction) and phase (3 levels: first, second and third anaesthetic) as the within-subjects factors. Blood pressure data collected from group i/nA (during the first

anaesthetic) and group p/fA were compared using repeated measures ANOVA with sample (2 levels: 1hr and 2hr post-induction) as the within-subjects factor and group (2 levels) as the between-subjects factor. Blood gas data collected from group i/nA (during the first anaesthetic in Experiment 2a) and group p/fA (during Experiment 2b) were compared using independent sample t-tests.

Baseline behavioural data were compared using repeated measures ANOVA, with day of testing (6 levels: testing days 1-6) and block (3 levels: first, middle and last block of 50 trials) as the within-subjects factors and future anaesthesia group (2 levels) as the between-subjects factor. Post-anaesthesia behavioural data for Experiment 2a were compared using repeated measures ANOVA, with phase (3 levels post first, second and third exposure to anaesthesia) day of testing (6 levels: testing days 1-6), and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors and group (2 levels) as the between-subjects factor. Post-anaesthesia behavioural data for Experiment 2b were compared using repeated measures ANOVA, with phase (2 levels pre and post-anaesthesia) day of testing (6 levels: testing days 1-6), and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors.

Post-lesion behavioural data for Experiment 2c were compared using repeated measures ANOVA, with phase (2 levels: pre and post-lesion), day of testing (6levels: testing days 1-6), and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors and group (2 levels) as the between subjects factor. As sample sizes were slightly different, Gabriel's post hoc analysis was performed. Baseline data were analysed separately to post-anaesthesia data for Experiments 2a and 2c.

Results

Experiment 1a: effect of anaesthetic exposure on performance of the SAT

Baseline behavioural testing performance

Performance of the task on the day preceding exposure to anaesthesia was taken as a measure of baseline performance. The baseline performance of rats did not differ depending on group allocation - as would be expected as groups were counterbalanced for performance. Specifically, rats assigned to receive anaesthesia did not differ from controls on signal detection or correct rejections. For all rats, the percentage of signals detected and the percentage of correct rejections made on non-signal trials decreased with decreasing signal duration and was increased in the middle block of trials as compared to the first or last block (see Fig 4.2 for baseline signal detection performance and Fig 4.5 for baseline correct rejection performance). Rats assigned to receive anaesthesia did not differ from controls on either signal trial omissions or non- signal trial omissions. Below are the supporting statistical analyses for these results.

Signal detection and correct rejections were analysed using repeated measures ANOVA with block (3 levels: first, middle and last block of 54 trials) as a within-subjects factor and anaesthesia group (3 levels: control (n=9), isoflurane plus nitrous oxide (n=8) and propofol plus fentanyl anaesthesia (n=9)) as the between-subjects factor. Signal duration (3 levels: 500, 100 and 25 ms) was also included as a within-subjects factor for signal detection.

Baseline performance on signal trials

Rats assigned to receive anaesthesia did not differ from controls on signal detection or correct rejections. There was no effect of anaesthesia group on signal detection ($F[2,23] = 0.037$, $p = 0.96$) or correct rejections ($F[2,23] = 0.109$, $p = 0.897$). There were no interactions of anaesthesia group with other factors and no higher order interactions (all p values > 0.074).

Baseline performance on non-signal trials

For all rats, the percentage of signals detected and the percentage of correct rejections made on non-signal trials decreased with decreasing signal duration and was increased in the middle block of trials as compared to the first or last block. As expected, the performance of all rats deteriorated with decreasing signal duration (mean percentage correct signal detection +/- sem at 500, 100 and 25 ms: 91 +/- 1.5, 77 +/- 2.5 and 53 +/- 3.9 respectively; all pairwise comparisons $p < 0.005$; see below for supporting statistical analysis). Additionally, the ability of all rats to correctly reject non-signal trials improved in the middle block of trials as compared to the preceding and following blocks (mean percentage of correct rejections +/- sem during first, second and last block of trials: 81 +/- 2, 86 +/- 1.5 and 82.7 +/- 1.9 respectively, pairwise comparisons of block 2 with block 1 and block 3: $p = 0.011$ and $p = 0.04$ respectively; see below for supporting statistical analysis).

There was a main effect of signal length on signal detection ($F[2,46] = 87.85$, $p < 0.005$) (Fig 4.2) that did not interact with group ($F[4,46] = 0.35$, $p = 0.84$) and a main effect of block on correct rejections ($F[2,46] = 3.252$, $p = 0.048$) that did not interact with group ($F[4,46] = 1.376$, $p = 0.257$). There were no higher order

interactions (all F values < 2.21, all p values > 0.074). The main effect of block reflected improved performance in the middle block of trials as compared to the preceding and following blocks (mean percentage of correct rejections +/- sem during first, second and last block of trials: 81 +/- 2, 86 +/- 1.5 and 82.7 +/- 1.9 respectively, pairwise comparisons of block 2 with block 1 and block 3: p = 0.011 and p = 0.04 respectively). It is not clear why the ability of rats to correctly reject non-signal trials improved in the middle of three blocks of trials as there was no difference between the three blocks. If anything it might have been expected that performance would deteriorate across blocks as rats became satiated but this was not the case.

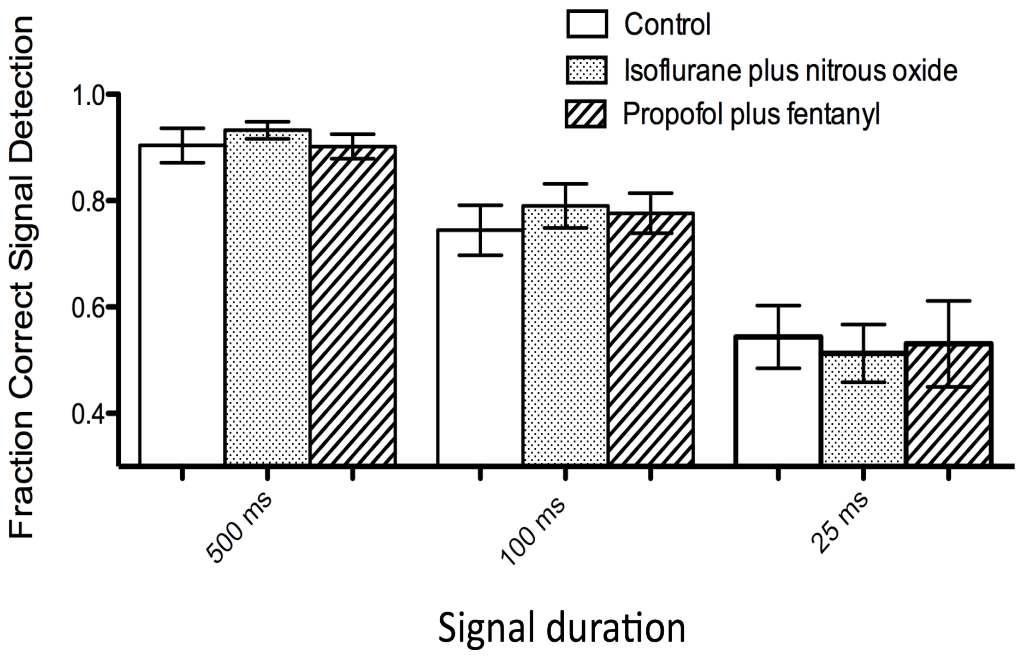

Figure 4.2 Baseline fraction of signal detections (equal to the percentage value divided by 100) for control rats (Group C), rats anaesthetised with isoflurane plus nitrous oxide (Group i/n) and rats anaesthetised with propofol plus fentanyl (Group p/f). Data for the three signal durations (500, 100 and 25 ms) are presented separately. Data are presented as mean +/- sem.

Baseline omissions

Rats assigned to receive anaesthesia did not differ from controls on either signal trial omissions or non-signal trial omissions. Many rats (from each of the three groups) made no omissions during sessions and therefore the number of omitted trials was averaged across day, signal duration (for signal trial omissions) and testing block for each rat, resulting in a single figure for each of the omission parameters per rat. Data were found to be non-normally distributed. Homogeneity of variance was tested using a one way ANOVA with absolute difference (the difference between the rank and the mean of rank for each data point) as the dependent variable and anaesthetic group as the between-subjects factor. Group variances were found to be equal for signal omissions and non-signal omissions (both F values < 0.17; both p values > 0.83). As group variances were equal, data also satisfied the requirement of the Kruskal-Wallis test for similarly shaped distributions and therefore a Kruskal-Wallis test was used to analyse baseline omissions data. Signal and non-signal trial omissions were analysed separately. There was no group difference in signal trial omissions ($H[2] = 0.638$, $p = 0.727$) or non-signal trial omissions ($H[2] = 0.102$, $p = 0.950$).

Anaesthesia

Rats that received propofol plus fentanyl were mildly acidaemic (low blood pH) compared to rats that were anaesthetised with isoflurane and nitrous oxide. There were no group differences in blood pressure or oxygen content of the blood. Statistical analyses below confirmed these impressions (see Table 4.4 for average physiological parameters of anaesthetised rats).

Blood pressure data collected from rats anaesthetised with isoflurane plus nitrous oxide (Group i/n) and from rats anaesthetised with propofol plus fentanyl (Group p/f), were compared using repeated measures ANOVA with sample (2 levels: 1hr and 2hr post-induction) as the within-subjects factor and group (2 levels) as the between-subjects factor. There were no main effects of group ($F[1,16] = 0.008$, $p = 0.93$) or sample ($F[1,16] = 0.387$, $p = 0.54$), and no group by sample interaction ($F[1,16] = 0.018$, $p = 0.89$) for blood pressure data from the two anaesthetic groups.

Blood gas data from the two anaesthetic groups were compared using independent samples t-tests. Rats anaesthetised with propofol plus fentanyl had a lower pH ($p < 0.005$) and higher pCO_2 ($p < 0.005$) at the end of the two-hour anaesthetic period, however there was no difference in pO_2 between the two groups ($p = 0.605$).

Parameter	**Group i/n (n = 8)**	**Group p/f (n = 9)**
Mean arterial blood pressure 1hr after induction of anaesthesia (mmHg)	109 +/- 6.7	110 +/- 4.5
Mean arterial blood pressure 2hr after induction of anaesthesia (mmHg)	112 +/- 5	112 +/- 4
pH	7.40 +/- 0.01	7.30 +/- 0.01**
pCO_2 (mmHg)	43 +/- 2	66 +/- 3**
pO_2 (mmHg)	103 +/- 8	108 +/- 6

*Table 4.4: Average physiological parameters for rats anaesthetised with a single episode of isoflurane plus nitrous oxide (Group i/n) or propofol plus fentanyl (Group p/f) for Experiment 1a. Rats that received propofol and fentanyl were acidaemic and hypercapnic compared to those that received isoflurane and nitrous oxide. Values are presented as mean +/- sem. **p < 0.01*

Post-anaesthetic behavioural testing

The following parameters were analysed; the percentage of signals detected (hits / hits plus misses, multiplied by 100), the percentage of correct rejections (correct rejections / correct rejections plus false alarms, multiplied by 100), number of omitted signal trials and number of omitted non-signal trials, for each session.

Signal detection post-anaesthesia

There was no effect of anaesthesia on signal detection; in other words, exposure to anaesthesia did not impair ability to detect signals whilst performing the SAT (see Fig 4.3 for post-anaesthesia signal detection performance; and below for statistical analysis).

The percentage of correct signal detections for each signal length (500, 100 and 25 ms), per session, was calculated (number of hits / hits plus misses, multiplied by 100) and analysed. The average percentage of signals detected by Group C, Group i/n and Group p/f was 74 +/- 3.2, 68.4 +/- 3.4 and 71.5 +/- 3.2 respectively.

Repeated measure analysis of variance (ANOVA) was used with day (6 levels: testing days 1-6), block (3 levels: first, middle and last block of 54 trials) and signal length (3 levels: 500, 100 and 25 ms) as within-subject factors and anaesthesia group (3 levels: control, isoflurane plus nitrous oxide anaesthesia and propofol plus fentanyl anaesthesia) as the between-subjects factor. There was no effect of anaesthesia group on signal detection ($F[2,23] = 0.738$, $p = 0.49$) and no interaction of group with other factors (all F values < 1.16; all p values > 0.3). There was no main effect of testing day and no interaction of day with other factors (all F values < 1.18; all p values > 0.31).

There was a main effect of block ($F[2,46] = 13.47$, $p < 0.005$), a main effect of signal length ($F[2,46] = 321.9$, $p < 0.005$) and an interaction of block with signal length ($F[4,92] = 3.25$, $p = 0.015$).

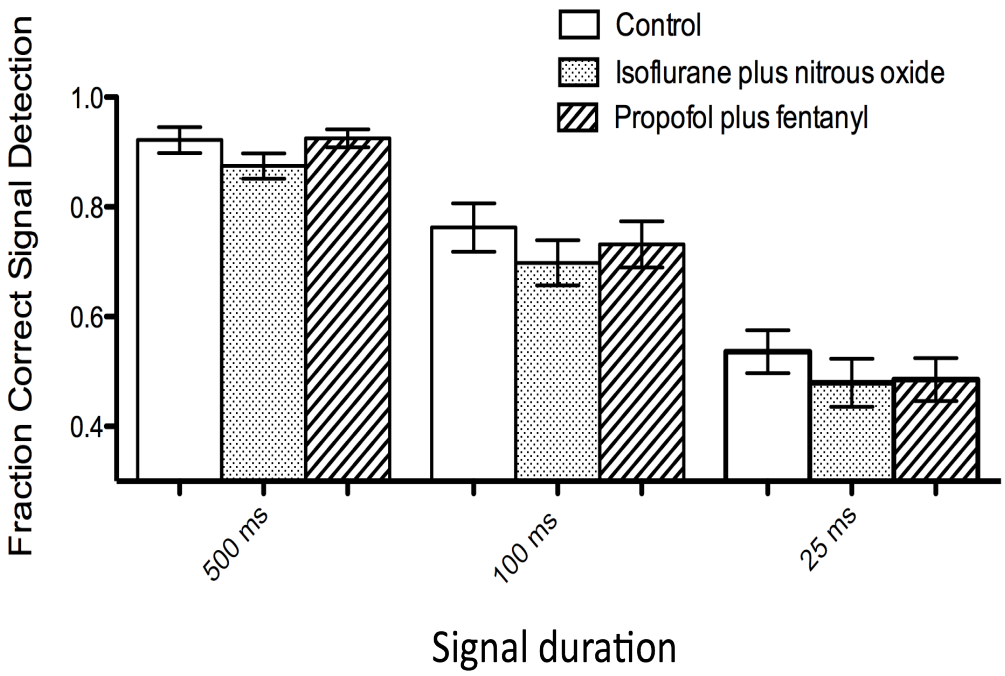

Figure 4.3 Post-anaesthesia fraction of signal detections (equal to the percentage value divided by 100) for control rats (Group C), rats anaesthetised with isoflurane plus nitrous oxide (Group i/n) and rats anaesthetised with propofol plus fentanyl (Group p/f). Data for each of the three signal durations (500, 100 and 25 ms) are presented separately. Data are presented as mean +/- sem.

Pairwise comparisons for the effect of block revealed that this effect was predominantly due to a decrease in performance during the middle block of trials (mean percentage of correct signal detections +/- sem: 70.6 +/- 1.9) compared to the first block (74.2 +/- 1.8, $p = 0.001$). The effect of signal duration on signal detection was due to performance decreasing as signal duration decreased, as expected (mean percentage of correct signal detections +/- sem for 500 ms signals 90.7 +/- 1.2; 100 ms signals 73.1 +/- 2.5 and 25 ms signals 50.1 +/- 2.3; all pairwise comparisons $p < 0.005$) (see Fig 4.4 for percentage correct signal detection values for each block and each signal duration, averaged across testing day and anaesthesia group).

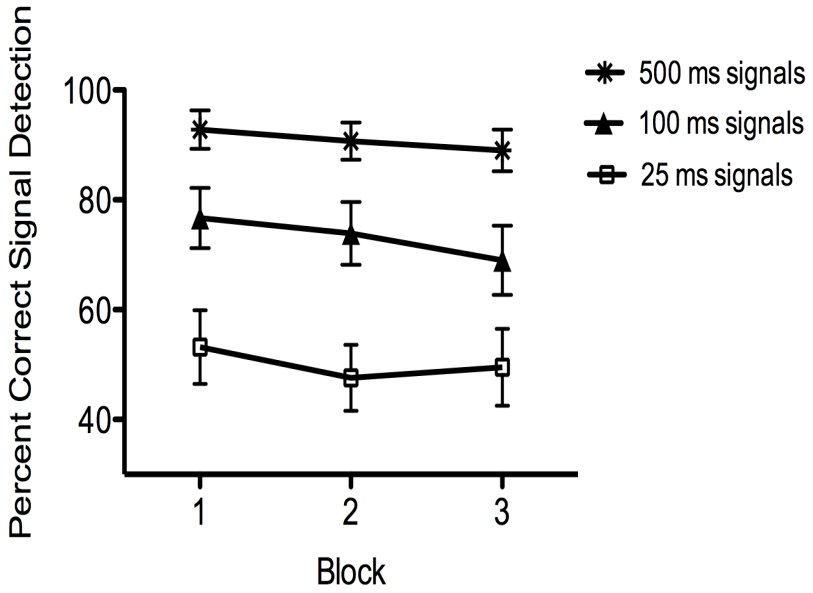

Fig 4.4 Percentage of correct signal detections for each of the three blocks of 54 trials within a session, presented separately for each signal length. Data are presented as mean +/- sem.

Correct rejections

There was no effect of anaesthesia on the percentage of correct rejections, in other words, exposure to anaesthesia did not impair ability to reject non-signal trials whilst performing the SAT (see Fig 4.5). As a measure of performance during non-signal trials, the percentage of trials during which the rat correctly identified absence of signal (number of correct rejections / number of correct rejections plus number of false alarms, multiplied by 100) was recorded and analysed. The average percentage of correct rejections for Group C, Group i/n and Group p/f was 84.5 +/- 1.6, 85.2 +/- 1.7 and 87.4 +/- 1.6 respectively.

Repeated measures ANOVA was used with day (6 levels: testing days 1-6) and block (3 levels: first, middle and last block of 54 trials) as within-subjects factors and anaesthetic group (3 levels) as the between-subjects factor.

There were no main effects of group ($F[2,23] = 0.91$, $p = 0.42$) or block ($F[2,46] = 2.036$, $p = 0.142$) on correct rejections. The performance across testing day increased but did not reach statistical significance ($F[5,115] = 2.078$, $p = 0.073$; mean percentage of correct rejections on day 1 83.4 +/- 1.7 and day 6 86.7 +/- 1.1); suggesting that the study was underpowered to detect changes in performance across day.

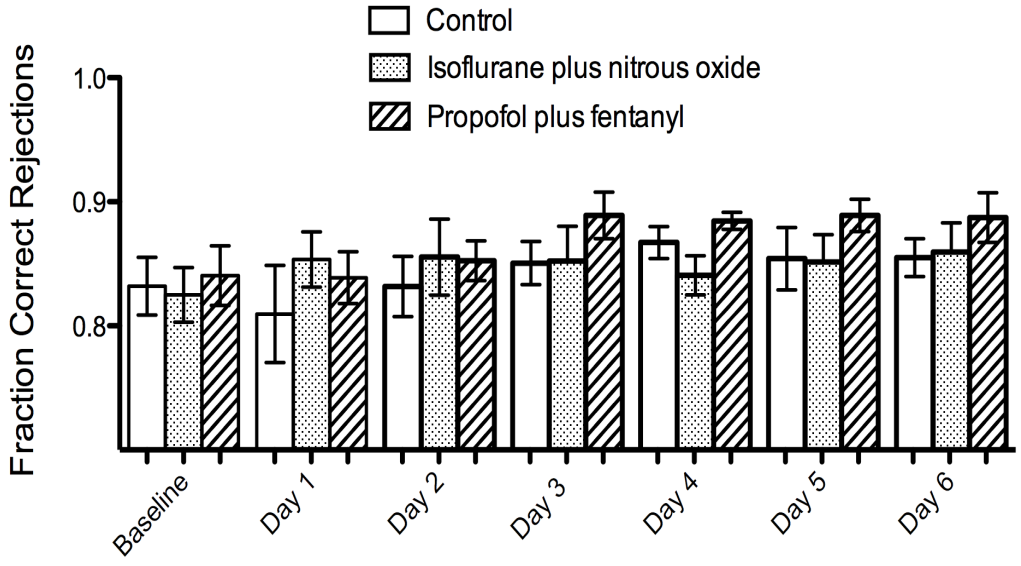

Figure 4.5 Fraction of correct rejections (equal to the percentage value divided by 100) at baseline and each post- anaesthesia day (days 1-6), for control rats (Group C), rats anaesthetised with isoflurane plus nitrous oxide (Group i/n) and rats anaesthetised with propofol plus fentanyl (Group p/f). Data are presented as mean +/- sem.

Omissions on signal and non-signal trials

The average number of omitted signal trials per session for Group C, Group i/n and Group p/f was 0.049 +/- 0.108, 0.33 +/- 0.115 and 0.091 +/- 0.108 respectively. The average number of omitted non-signal trials per session for Group C, Group i/n and Group p/f was 0.64 +/- 0.08, 1.6 +/- 0.69 and 0.60 +/- 0.09 respectively. There were no group differences in the number of signal trial omissions or the number of non-signal trial omissions.

Many rats (from each of the three groups) made no omissions during sessions and therefore the number of omitted trials was averaged across *all* testing blocks for each rat, resulting in a single figure for each of the omission parameters per rat. Data were found to be non-normally distributed (D[26] values for both signal trial omissions and non-signal trial omissions <0.005). Homogeneity of variance was tested using a one-way ANOVA with absolute difference (the difference between the rank and the mean of rank for each data point) as the dependent variable and anaesthetic group as the between-subjects factor. Group variances were found to be equal for signal omissions and non-signal omissions (both F values < 0.237; both p values > 0.79). As group variances were equal, data also satisfied the requirement of the Kruskal-Wallis test for similarly shaped distributions and therefore a Kruskal-Wallis test was used to analyse baseline omissions data. Signal and non-signal trial omissions were analysed separately. There was no group difference in signal trial omissions ($H[2] = 2.19$, $p = 0.334$) or non-signal trial omissions ($H[2] = 2.08$, $p = 0.354$).

Experiment 1b - effect of scopolamine on performance of the SAT

Behavioural testing performance

Signal detection

Repeated measures ANOVA was used with dose (3 levels: vehicle, low dose scopolamine and high dose scopolamine), block (3 levels: first, middle and last block of 54 trials) and signal duration (3 levels: 500, 100 and 25 ms) as the within-subjects factors and prior anaesthesia group (3 levels: control (n=9), isoflurane plus nitrous oxide (n=8) and propofol plus fentanyl (n=8)) as the between-subjects factor. Gabriel post hoc analysis was performed.

Prior anaesthesia treatment did not alter the response to scopolamine on performance in the sustained attention task (Prior anaesthesia: $F[2,22] = 1.13$, $p = 0.341$; all interactions $p > 0.1$). The administration of scopolamine impaired the detection of signals in all subjects (Dose: $F[2,44] = 28.14$, $p < 0.001$), an effect that interacted with signal duration ($F[4,88] = 22.41$, $p < 0.001$). This effect was most pronounced for 500 ms signals with impairments in detection of this signal length with both the low (vehicle vs. 0.1 mg/kg: $p < 0.001$) and the high dose (vehicle vs. 0.25 mg/kg: $p < 0.001$). The higher dose of the drug also impaired detection of the 100 ms signal ($p < 0.001$) while the lower dose was without effect at this signal length ($p = 0.17$). Neither dose of the drug impacted detection of the 25 ms signal (all $p > 0.29$). Although this result may seem counterintuitive, it is proposed that the lack of effect of scopolamine on performance of the short duration signals is due to a floor effect (i.e. performance is already so low during the 25 ms trials, because attentional demand is high, that no further reduction in performance is seen with the addition of scopolamine). There were no other significant interactions in the analyses of the scopolamine data (all $p > 0.16$).

Correct rejections

The effect of scopolamine on the number of correct rejections did not depend on prior anaesthesia treatment (Prior anaesthesia group: $F[2,22] = 0.463$, $p = 0.635$; all interactions $p > 0.37$). The administration of scopolamine impaired the rejection of non-signal trials (Dose: $F(2,44) = 17.50$, $p < 0.001$). Both the low (vehicle vs. 0.1 mg/kg: $p < 0.001$) and the high dose (vehicle vs. 0.25 mg/kg: $p < 0.001$) of scopolamine impaired rejection of non-signal trials but there was no difference between the two doses (0.1 mg/kg vs. 0.25 mg/kg: $p = 0.21$).

Experiment 2a: effect of repeated administration of isoflurane plus nitrous oxide on performance of the 5CSRTT

Anaesthetic physiology

Body temperature was successfully maintained between 36.5C and 37.5C in all anaesthetised rats. In order to investigate a potential cumulative effect of anaesthesia on blood pressure, blood pressure data were compared using repeated measures ANOVA, with phase (3 levels: 1st, 2nd and 3rd anaesthetic) and sample (2 levels: 1hr and 2hr after induction) as the within-subjects factors. There were no main effects of phase ($F[2,16] = 0.744$, $p = 0.491$) or sample ($F[1,8] = 0.487$, $p = 0.51$) and no interaction of phase and sample ($F[2,16] = 0.985$, $p = 0.395$) (see Table 4.5 for average physiological parameters for anaesthetised rats).

In order to investigate a potential cumulative effect of anaesthesia on blood gas parameters, blood gas data were compared using repeated measures ANOVA, with phase (3 levels: 1st, 2nd and 3rd anaesthetic) as the within-subjects factor. There was no effect of phase on blood pH ($F[2,16] = 0.32$, $p = 0.731$), pCO_2 ($F[2,16] = 0.43$, $p = 0.658$) or pO_2 ($F[2,16] = 0.344$, $p = 0.714$).

Parameter	First anaesthetic exposure (n=9)	Second anaesthetic exposure (n=9)	Third anaesthetic exposure (n=9)
Mean arterial blood pressure 1hr after induction of anaesthesia (mmHg)	101.7 +/- 4	106.2 +/- 4.2	104.8 +/- 3.7
Mean arterial blood pressure 2hr after induction of anaesthesia (mmHg)	102.7 +/- 2.8	104 +/- 4.2	112.6 +/- 4.4
pH	7.37 +/- 0.02	7.38 +/- 0.01	7.36 +/- 0.01
pCO_2 (mmHg)	47.9 +/- 1.6	46.1 +/- 1.1	47 +/- 1.8
pO_2 (mmHg)	117.8 +/- 8.7	112.3 +/- 4.4	117.9 +/- 4.3

Table 4.5: Average physiological parameters for rats anaesthetised with isoflurane plus nitrous oxide, in Experiment 2a, on each of three occasions separated by one week. Values are presented as mean +/- sem.

Behavioural testing

The following parameters were analysed; choice accuracy (a percent correct measure: correct / correct plus incorrect, multiplied by 100), omissions (a percent omitted measure: number of omitted trials / total number of trials, multiplied by 100), number of premature responses (nose pokes before stimulus presentation), number of perseverative responses (repeated nose pokes at the correct response window after a stimulus presentation), the response time (time taken to respond to a stimulus) and the reward collection latency (time taken to collect the food reward).

Baseline 5CSRTT performance

Baseline performance consisted of six days of testing, the last of which occurred 24hrs before the first anaesthetic exposure. The baseline performance of rats did not differ depending on group allocation - as would be expected as groups were counterbalanced for performance. The performance of all rats deteriorated during the middle block of trials, indicating that attentional demand was increased during the high event rate block - as was expected.

As some rats made zero omissions on some days, data for omissions were averaged across day, resulting in three omission values per rat (one for each block of 50 trials). Data were analysed using repeated measures ANOVA with day (6 levels: testing days 1-6; not for analysis of omissions) and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors and anaesthesia group (2 levels: control (n=8) and isoflurane plus nitrous oxide (n=9)) as the between-subjects factor. There was no effect of anaesthesia group on choice accuracy ($F[1,15] = 1.787$, $p = 0.2$), omissions ($F[1,15] = 2.07$, $p = 0.17$), premature responses ($F[1,15] = 0.09$, $p = 0.77$), perserverative responses ($F[1,15] = 0.01$, $p = 0.925$), response latency ($F[1,15] = 0.004$, $p = 0.953$) or reward collection latency ($F[1,15] = 0.147$, $p = 0.71$). There were no interactions of anaesthesia group with other factors and no higher order interactions (all p values > 0.12).

There was a main effect of block on choice accuracy ($F[2,30] = 7.85$, $p = 0.002$) and omissions ($F[2,30] = 32.27$, $p < 0.005$). This effect reflected a decrease in accuracy for all rats during the high event rate (middle) block of trials (mean +/- sem: 75.24 +/- 2.1), relative to the preceding block (80.63 +/- 2.4; middle vs first: $p = 0.001$) that did not recover in the following block (76.51 +/- 1.6; middle vs last: $p = 0.41$) when the low event rate was reintroduced. There was also an increase in omissions for all rats during the high event rate (middle) block of trials (mean +/- sem: 15.5 +/- 1.7), relative to the preceding (2.84 +/- 0.32; middle vs first: $p < 0.005$) and the following (6.36 +/- 1.49; middle vs last: $p < 0.005$) blocks (see Fig 4.6 for a representation of average baseline (pre-anaesthesia) choice accuracy data, and Fig 4.7 for a representation of average baseline (pre-anaesthesia) omissions data).

Post-anaesthesia choice accuracy

Accuracy was assessed by analysing a percent correct measure (number of correct choices / number of correct choices plus number of incorrect choices, multiplied by 100). All rats demonstrated decreased performance during high event rate (middle block) trials (mean +/- sem control: 76.86 +/- 2.9; anaesthetised: 72.1 +/- 2.76) relative to the preceding block (control: 83.53 +/- 3.1; anaesthetised: 76.73 +/- 2.91), that did not recover in the following block (control: 76.78 +/- 2.88; anaesthetised: 72.76 +/- 2.72). This reflected the fact that rats made more errors (nose poked the wrong response window) when the event rate was increased - indicating that attentional demand was increased during the high event rate block. This effect was not exacerbated by anaesthesia (see below for statistical analysis of choice accuracy data - presented separately for effects of testing day and anaesthesia group).

Effect of testing day (days 1-6) on accuracy

There was no effect of testing day within a testing phase on the ability of rats to accurately respond to the stimulus.

Repeated measure analysis of variance (ANOVA) was used with test phase (3 levels: post 1st, 2nd and 3rd anaesthetic), day (6 levels: 6 days of testing) and block (3 levels: first, middle and last blocks of 50 trials) as within-subjects factors and anaesthesia group (2 levels: control and anaesthesia) as the between-subjects factor. There was no main effect of day ($F[5,75] = 0.298$, $p = 0.912$), no interaction of testing day with anaesthesia group ($F[5,75] = 0.717$, $p = 0.613$) and no higher order interactions of day with phase and / or block and anaesthesia group (all p values > 0.35).

Effect of anaesthesia group

There was no effect of anaesthesia on choice accuracy, in other words, exposure to anaesthesia did not impair accurate performance of the 5CSRTT.

Repeated measure ANOVA with test phase (3 levels: pre-anaesthesia, post 1st, 2nd and 3rd anaesthetic) and block (3 levels: first, middle and last blocks of 50 trials) as the within-subjects factors and anaesthetic group (2 levels) as the between-subjects factor revealed a main effect of block ($F[2,30]=13.63$, $p<0.0005$). This effect reflected a decrease in accuracy in all rats during the high event rate (middle) block of trials (mean +/- sem: 74.46 +/- 2.01), relative to the preceding block (80.13 +/- 2.12; middle vs first: $p = 0.002$) that did not recover in the following block (74.77 +/- 2.0; middle vs last $p < 0.0005$). This effect did not interact with anaesthesia group ($F[2,30] = 0.689$, $p = 0.51$) and there was no main effect of anaesthesia group ($F[1,15] = 1.85$, $p = 0.193$), or interaction of group with any other factors (all p values > 0.4). Figure 4.6 shows average percentage correct choices (choice accuracy) for control rats and anaesthetised rats, during the pre-anaesthesia and each of the three post-anaesthesia phases (Post1, Post2 and Post3).

These analyses indicate that there were no anaesthesia-induced impairments in choice accuracy.

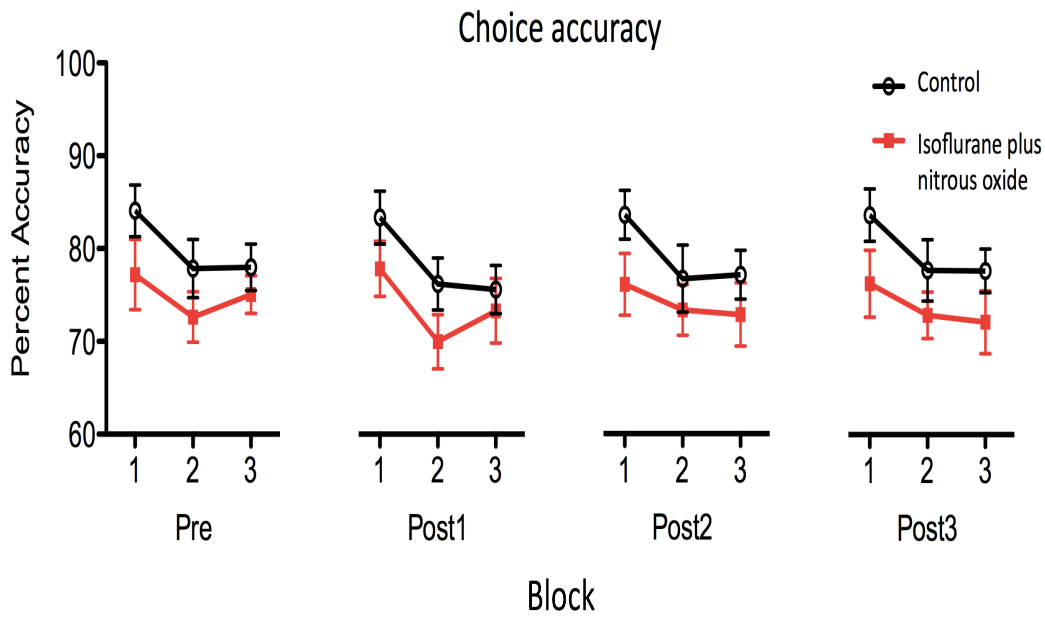

Figure 4.6 Percentage of correct choices (choice accuracy) for control rats and rats that received isoflurane plus nitrous oxide anaesthesia. Data are presented as mean +/- sem for each block of 50 trials (blocks 1-3) within a session, during the pre-anaesthesia phase (Pre) and each of the three post-anaesthesia phases (Post1, Post2 and Post3). Please note that statistical analysis of post-anaesthetic data included three phases only (Post 1, Post 2 and Post 3).

Omissions

As some rats made zero omissions on some days, data for omissions were averaged across day for each phase separately, resulting in three omission values per rat (one for each block of 50 trials) per phase (1st, 2nd and 3rd anaesthetic) - nine values per rat in total.

Data were analysed using repeated measures ANOVA with phase (3 levels: 1st, 2nd and 3rd anaesthetic) and block (3 levels: first, middle and last block of 50 trials) as the within-subjects factors and anaesthesia group (2 levels) as the between-subjects factor. All rats demonstrated an increase in omissions during the high event rate (middle block) of trials (mean +/- sem control: 10.73 +/- 2.4; anaesthetised 16.33 +/- 2.3) relative to the preceding block (control: 3.83 +/- 0.68; anaesthetised: 4.52 +/- 0.64)

or following block (control: 4.96 +/- 0.97; anaesthetised 4.68 +/- 0.91). This effect was exacerbated after the first and second anaesthesia exposures, but rats in the anaesthesia group appeared to become resistant to this effect following the subsequent anaesthesia exposure (see below for statistical analysis of omission data).

Effect of anaesthesia group

Rats that had been anaesthetised omitted more trials in the high event rate (middle) block of trials than control rats, during the testing phase after the first and second exposures to anaesthesia. Repeated measure ANOVA with test phase (3 levels: pre-anaesthesia, post 1st, 2nd and 3rd anaesthetic) and block (3 levels: first, middle and last blocks of 50 trials) as within-subjects factors and anaesthetic group (2 levels) as the between-subjects factor revealed main effects of phase ($F[2,30]=4.63$, $p=0.018$), block ($F[2,30]=36.49$, $p<0.0005$) and an interaction of phase and block ($F[4,60]=3.23$, $p=0.018$). These effects reflect an increase in omissions in all rats during the high event rate (second) block, relative to the preceding and following block, the magnitude of which varies depending on the testing phase. There was no main effect of anaesthesia group ($F[1,15] = 1.92$, $p = 0.187$). However, critically, there was an interaction of phase, block, and anaesthesia group ($F[4,60]=5.42$, $p=0.001$), indicating that the phase x block interaction differed depending on whether rats were exposed to anaesthesia.

Decomposition of the interaction between phase, block and anaesthesia group

This interaction was decomposed by examining the significance of the group x block interaction in each of the three testing phases. Repeated measures ANOVAs were constructed for each testing phase separately. The group x block interaction was

significant in the Post 1st anaesthetic exposure and Post 2nd anaesthetic exposure phases ($F[2,30]=4.94$, $p=0.014$ and $F[2,30]=3.53$, $p=0.042$, respectively) but not for the Post 3rd anaesthetic exposure phase ($F[2,30] = 0.184$, $p = 0.83$). Figure 4.7 shows average percentage omissions for control rats and anaesthetised rats.

Taken together, these analyses indicate a specific increase in omissions during conditions of high attentional demand in rats exposed to anaesthesia during the test period after the first and second exposure, but this effect is not seen with the subsequent third exposure, presumably either because increased practice with the task masks anaesthesia-induced impairments in attention or because rats somehow become resistant to the effect of anaesthesia on attention.

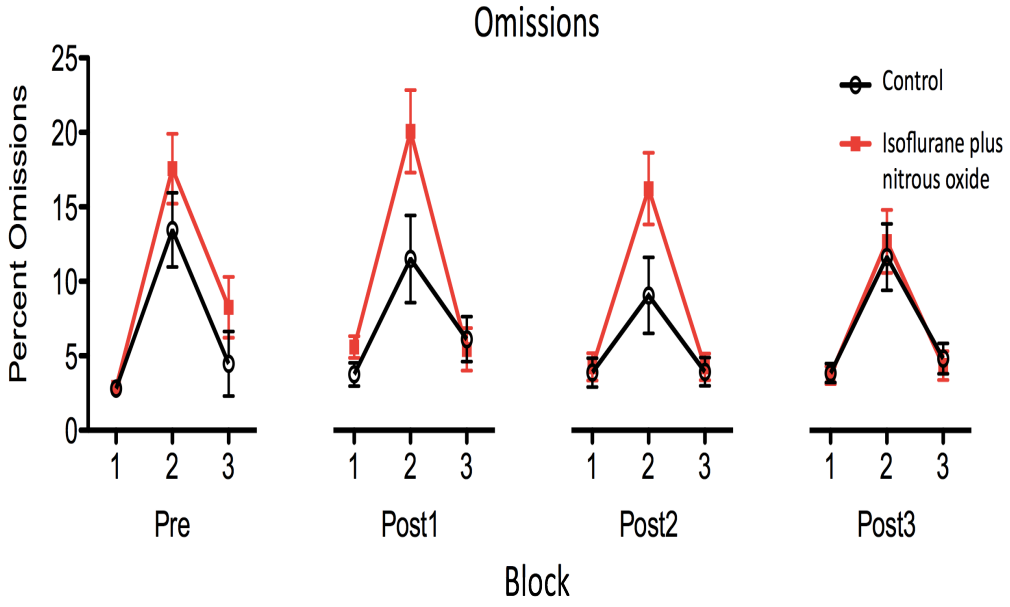

Fig 4.7 Percentage omissions for control rats and rats that received isoflurane plus nitrous oxide anaesthesia. Data are presented as mean +/- sem for each block of 50 trials (blocks 1-3), during the pre-anaesthesia phase (Pre) and each of the three post-anaesthesia phases (Post1, Post2 and Post3). Please note that statistical analysis of post-anaesthetic data included three phases only (Post 1, Post 2 and Post 3).

Premature responses

Exposure to isoflurane plus nitrous oxide anaesthesia was associated with a numerical increase in premature responses, made before signal presentation, that did not reach statistical significance (mean premature responses +/- sem control: 1.84 +/- 1.15; anaesthesia: 4.59 +/- 1.1).

As some rats made zero premature responses on some days, data for premature responses were averaged across day for each phase separately. The number of premature (anticipatory responses made before the onset of the stimulus) responses was analysed using repeated measures ANOVA with phase (3 levels: post 1st, 2nd and 3rd anaesthetic) and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors and anaesthesia group (2 levels) as the between-subjects factor. There was no effect of anaesthesia group on premature responses ($F[1,15] = 3.03$, $p = 0.102$) and no interactions of group with other factors (all p values > 0.33). The number of premature responses was similar across all post anaesthetic phases ($F[2,30] = 0.286$, $p = 0.753$) and across all blocks within a session ($F[2, 30] = 0.754$, $p = 0.48$).

Perseverative responses

Anaesthesia had no effect on the number of perseverative responses at the correct response window (mean perseverative responses +/- sem control: 27.26 +/- 5.2; anaesthesia: 29.83 +/- 4.9). The number of perseverative responses was analysed using repeated measures ANOVA with phase (3 levels: post 1st, 2nd and 3rd anaesthetic), day (6 levels: testing day 1-6) and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors and anaesthesia group (2 levels) as the between-subjects factor. There was no effect of anaesthesia group on perseverative responses (F[1,15]

= 0.129, p = 0.73) and no interactions of group with other factors (all p values > 0.17). The number of perseverative responses was similar across all post-anaesthetic phases (F[2,30] = 1.245, p = 0.302) and across all blocks within a session (F[2,30] = 0.402, p = 0.672).

Response time

Anaesthetised rats took longer to respond to the stimulus (mean response time +/- sem control: 1002.9 +/- 30.6 ms; anaesthesia: 1122.2 +/- 28.9 ms). The time taken to respond to the stimulus (time between stimulus presentation and nose poke) was analysed using repeated measures ANOVA with phase (3 levels: post 1st, 2nd and 3rd anaesthetic), day (6 levels: testing day 1-6) and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors and anaesthesia group (2 levels) as the between-subjects factor. There was a main effect of anaesthesia group (F[1,15] = 8.04, p = 0.013) that did not interact with other factors (all p values > 0.107). There was a trend towards an effect of day on response times (F[5,75] = 2.0, p = 0.087) but no day by group interaction (F[5, 75] = 1.426, p = 0.225) and no interaction of day with other factors (all p values > 0.32).

Reward collection latency

The amount of time taken to collect the food reward was similar for control rats (mean +/- sem 1.744 +/- 0.107 s) and anaesthetised rats (1.943 +/- 0.1 s). The time taken to collect the food reward (time between nose poke at response window and collection of food reward from food magazine) was analysed using repeated measures ANOVA with phase (3 levels: post 1st, 2nd and 3rd anaesthetic), day (6 levels: testing day 1-6) and block (3 levels: first, middle and last block of 50 trials) as within-subjects factors and

anaesthesia group (2 levels) as the between-subjects factor. There was no main effect of anaesthesia on reward collection latency ($F[1,15] = 1.832$, $p = 0.196$) and no interaction of anaesthesia with other factors (all p values > 0.19). There was a trend towards an effect of phase ($F[2,30] = 3.266$, $p = 0.052$) that reflected the trend that rats took longer to collect the food reward during the first phase (mean +/- sem 1.892 +/- 0.72 s) than during the second or third phases (1.809 +/- 0.077 s and 1.829 +/- 0.079 s respectively).

Experiment 2b: propofol plus fentanyl - effect of propofol plus fentanyl on performance of the 5CSRTT

Anaesthetic physiology

Body temperature was successfully maintained between 36.5C and 37.5C in all anaesthetised rats. In order to investigate a potential difference in physiological parameters between rats anaesthetised with a single exposure of isoflurane plus nitrous oxide in Experiment 2a and rats anaesthetised with propofol plus fentanyl in Experiment 2b, blood pressure data from the two experiments were compared using repeated measures ANOVA, with sample (2 levels: 1hr and 2hr after induction) as the within-subjects factor and anaesthesia group (2 levels: Group i/n from Experiment 2a and Group p/f from Experiment 2b) as the between-subjects factor. There was no group difference in blood pressure ($F[1,13] = 0.444$, $p = 0.517$). There was no difference between the first and second sample of blood pressure ($F[1,13] = 0.047$, $p = 0.833$) and no interaction between sample and group ($F[1,13] = 0.047$, $p = 0.833$).

Blood gas data from Experiment 2b was compared to that of Experiment 2a (see Table 4.5 for results of 2a) using independent samples t-tests. Rats anaesthetised with propofol plus fentanyl were moderately acidaemic compared to those anaesthetised with isoflurane plus nitrous oxide from Experiment 2a (mean pH +/- sem propofol plus fentanyl: 7.2 +/- 0.01, isoflurane plus nitrous oxide: 7.37 +/- 0.02 $p = < 0.005$). Rats anaesthetised with propofol plus fentanyl were also relatively hypercapnic (mean pCO_2 +/- sem propofol plus fentanyl: 62 +/- 5.8, isoflurane plus nitrous oxide: 47.9 +/- 1.6 $p < 0.005$). There was no difference in blood oxygen content between rats in the two experiments (mean pO_2 +/- sem propofol plus fentanyl: 119 +/- 19.2, isoflurane plus nitrous oxide: 117.8 +/- 8.7 $p = 0.924$).

Behavioural testing

The following parameters were analysed: choice accuracy (a percent correct measure: correct / correct plus incorrect, multiplied by 100), omissions (a percent omitted measure: number of omitted trials / total number of trials, multiplied by 100), number of premature responses (nose pokes before stimulus presentation), number of perseverative responses (repeated nose pokes at the correct response window after a stimulus presentation), the response time (time taken to respond to a stimulus) and the reward collection latency (time taken to collect the food reward).

Choice accuracy

Accuracy was assessed by analysing a percent correct measure (number of correct choices / number of correct choices plus number of incorrect choices, multiplied by 100). Anaesthesia did not affect choice accuracy (mean percentage of accurate choices

+/- sem pre anaesthesia: 77.66 +/- 1.47; post-anaesthesia 76.17 +/- 2.77). See below for statistical analysis of choice accuracy data.

Repeated measure ANOVA was used with anaesthesia phase (2 levels: pre and post-propofol plus fentanyl), day (6 levels: 6 days of testing) and block (3 levels: first, middle and last blocks of 50 trials) as the within-subjects factors. There was no effect of anaesthesia on choice accuracy ($F[1,5] = 0.131$, $p = 0.732$) and anaesthesia did not interact with day or block (p values > 0.39). There was a main effect of block ($F[2,10] = 7.75$, $p = 0.01$). As was seen in experiment 2a, this effect reflected that rats made less accurate choices during the high event rate (middle) block of trials (mean +/- sem 73.83 +/- 2.3) compared to the first block (81.92 +/- 2.1, middle vs first block $p = 0.006$) of trials, and this did not recover accuracy in the last block of trials (75.66 +/- 3, middle vs last block $p = 1.0$) (see Fig 4.8 for average percentage correct choices pre and post-anaesthesia).

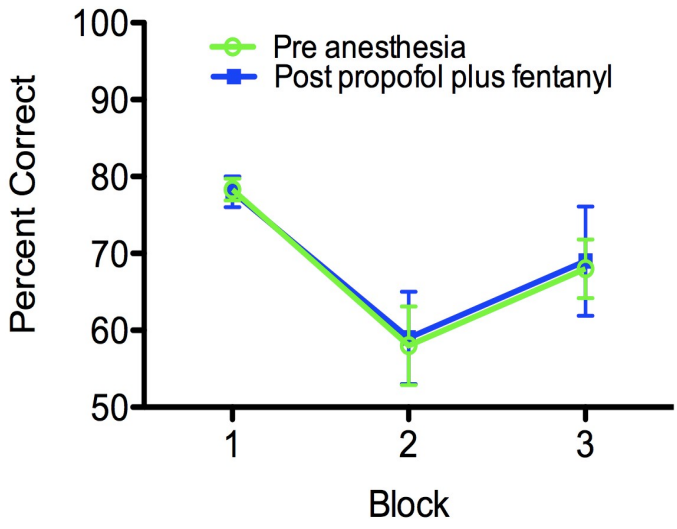

Figure 4.8 Percentage of correct choices (choice accuracy) for rats before (Pre) and after (Post 1) administration of a single episode of propofol plus fentanyl. Data are presented as mean +/- sem for each block of 50 trials (blocks 1-3).

Omissions

As some rats made zero omissions on some days, data for omissions were averaged across day for each phase (pre and post-anaesthesia), resulting in three omission values per rat (one for each block of 50 trials) per phase (pre and post-anaesthesia) - six values per rat in total. Anaesthesia did not affect the percentage of omitted trials (mean percentage of omissions +/- sem pre anaesthesia: 6.11 +/- 1.28; post-anaesthesia 5.57 +/- 1.21) (see below for statistical analysis).

Data were analysed using repeated measures ANOVA with anaesthesia phase (2 levels: pre and post-anaesthesia) and block (3 levels: first, middle and last block of 50 trials) as the within-subjects factors. There was no effect of anaesthesia on omissions ($F[1,5] = 0.12$, $p = 0.74$) and anaesthesia did not interact with block ($F[2,10] = 1.46$, $p = 0.28$). There was a main effect of block ($F[2,10] = 13.4$, $p = 0.001$. As was seen in Experiment 2a, this effect reflected that rats omitted more trials during the high event rate (middle) block of trials (10.5 +/- 1.97) compared to the first block (3.22 +/- 0.55, middle vs first: $p = 0.015$) or last block (3.96 +/- 0.97, middle vs last: $p = 0.05$) of trials (see Fig 4.9 for average percentage omissions pre and post-anaesthesia).

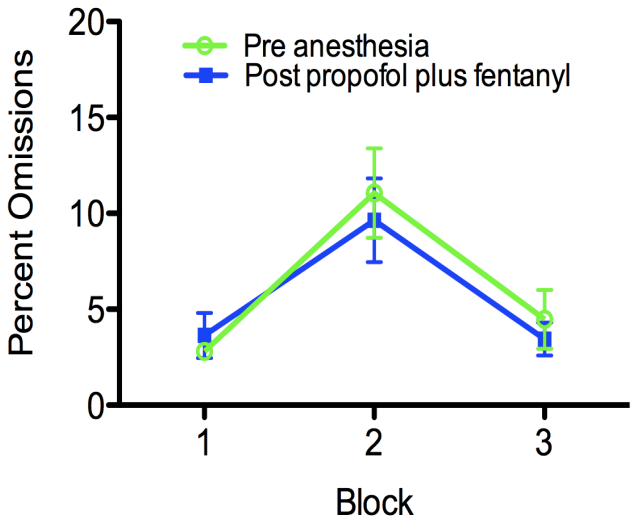

Figure 4.9 Percentage of omissions for rats before (Pre) and after (Post 1) administration of a single episode of propofol plus fentanyl. Data are presented as mean +/- sem for each block of 50 trials (blocks 1-3).

Premature responses

Many rats made zero premature responses (nose pokes before stimulus presentation) during both pre and post anaesthesia phases and therefore data were averaged across *all* trials within a phase (pre and post-anaesthesia), resulting in a single value for each phase per rat - two values per rat. Data were analysed using paired samples t-test. There was no effect of anaesthesia on premature responses (mean +/- sem pre anaesthesia: 0.97 +/- 0.17; post anaesthesia: 1.15 +/- 0.3; $p = 0.61$).

Response time

Propofol plus fentanyl anaesthesia did not affect time taken to respond to the stimulus (mean +/- sem pre anaesthesia: 1035.4 +/- 25.6 ms; post-anaesthesia: 1054.8 +/- 32.78).

Data were analysed using repeated measures ANOVA with anaesthesia phase (2 levels: pre and post-anaesthesia) and block (3 levels: first, middle and last block of

50 trials) as the within-subjects factors. There was no effect of anaesthesia on response time ($F[1,5] = 0.147$, $p = 0.72$) and anaesthesia did not interact with other factors (all p values > 0.21). There was a main effect of block ($F[2,10] = 408.94$, $p < 0.005$), that did not interact with other factors (all p values > 0.21). This effect reflected an increase in response time across testing blocks (mean +/- sem block1: 576.1 +/- 19.5; block 2: 1020.4 +/- 22.5; block 3: 1538.7 +/- 30.3, all p values < 0.005). The basis for this effect is not clear but it could reflect changes in motivation across the session.

Experiment 2c: basal forebrain lesions

Behavioural testing

The following parameters were analysed: choice accuracy (a percent correct measure: correct / correct plus incorrect, multiplied by 100) and omissions (a percent omitted measure: number of omitted trials / total number of trials, multiplied by 100). Rats that received a cholinergic basal forebrain lesion made less accurate choices (mean +/- sem sham: 72.56 +/- 3.95; lesion: 66.89 +/- 3.34) and made more omissions (mean +/- sem sham: 6.29 +/- 0.9; lesion: 11.76 +/- 0.76) than sham operated rats.

Data were analysed using repeated measures ANOVA with surgical phase (2 levels: before and after surgery), day (6 levels: 6 days of testing; accuracy only) and block (3 levels: first, middle and last blocks of 50 trials) as the within-subjects factors and surgical group (2 levels: sham and lesioned) as the between-subjects factor. Bonferroni post hoc analysis was performed.

Choice accuracy

There was no main effect of surgical group ($F[1,10] = 1.2$, $p = 0.298$) on choice accuracy. Choice accuracy increased across day, with a main effect of day ($F[5,50] = 6.16$, $p < 0.005$). As expected rats made less accurate choices during the high event rate (middle) block of trials, and so there was a main effect of block ($F[2,20] = 7.4$, $p = 0.004$). Rats made less accurate choices after surgery (main effect of phase ($F[1,10] = 21.15$, $p = 0.001$)) and this effect interacted with block ($F[2,20] = 5.17$, $p = 0.015$). As anticipated, the effect of phase also interacted with surgical group ($F[1,10] = 5.66$, $p = 0.039$). There was also a trend towards an interaction of phase, group and block ($F[2,20] = 3.31$, $p = 0.057$).

The interaction between phase and surgical group was decomposed by examining the effect of surgical group in each of the phases separately. Repeated measures ANOVAs were constructed for each of the phases (pre surgery and post-surgery) separately, with day (6 levels: testing day 1-6) and block (3 levels: first, middle and last block of 50 trials) as the within-subjects factors and surgical group (2 levels) as the between-subjects factor.

As expected there was a main effect of day ($F[5,50] = 3.47$, $p = 0.009$) and a main effect of block ($F[2,20] = 4.729$, $p = 0.021$ on pre-surgical (baseline) choice accuracy. Both would be sham operated rats and would be lesioned rats made a similar percentage of accurate choices before surgery (mean +/- sem sham: 75.8 +/- 3.82, lesion: 77.06 +/- 3.23; main effect of surgical group $F[1,10] = 0.063$, 0.806). There was, however, a trend towards an interaction between block and group ($F[2,20] = 3.394$, $p = 0.054$) that did not reach significance (see Fig 4.10 for a representation of

the average pre-surgical (baseline) values), suggesting that the groups were not sufficiently counterbalanced pre-surgery, for a study with this (relatively low) number of subjects.

There was also the expected main effect of day ($F[5,50] = 3.46$, $p = 0.009$) and block ($F[2,20] = 6.84$, $p = 0.005$) on post-surgical choice accuracy. Importantly, in the post-surgical phase lesioned rats made less accurate choices than sham rats (mean +/- sem sham: 69.33 +/- 5.15, lesion: 56.72 +/- 4.35; $F[1,10] = 4.5$, $p = 0.051$) (see Figure 4.10 for a representation of the average post-surgical values). This comparison did notquite reach statistical significance, most likely suggesting that it was underpowered, due to the relatively low number of subjects (lesion: n=7; sham n=5). However, the result does very strongly support that the behavioural testing apparatus was set up correctly and that the task was dependent on basal forebrain function, as intended (see omissions and immunohistochemistry below for further confirmation).

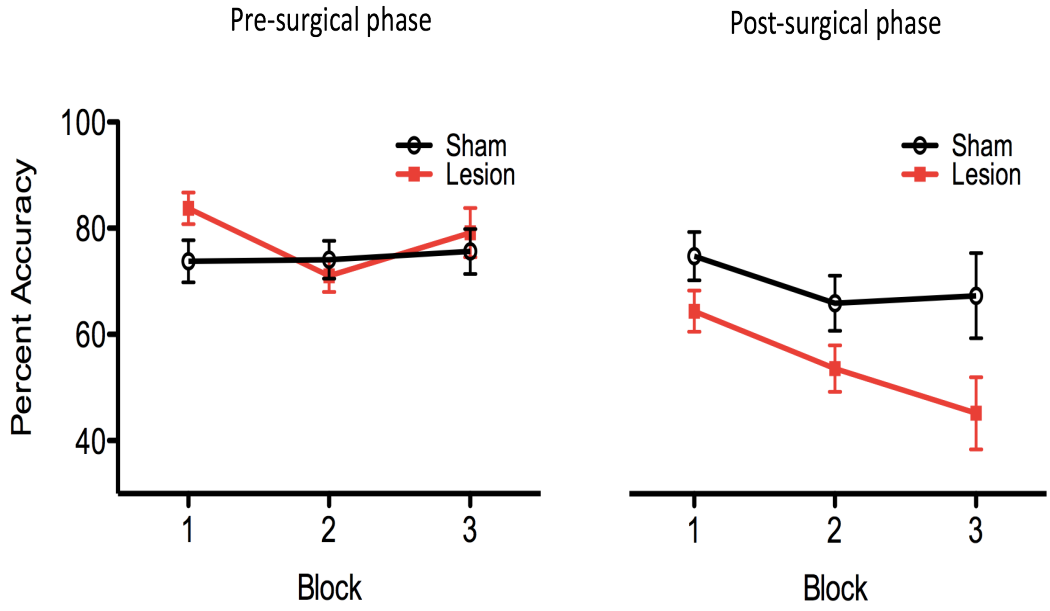

Figure 4.10 Percentage of accurate choices for sham operated rats and rats that received a cholinergic basal forebrain lesion, during the pre and post surgical phases. Data are presented as mean +/- sem for each block of 50 trials (blocks 1-3). Lesioned rats make less accurate choices, than sham operated rats, in the post-surgical phase.

Omissions

As some rats made zero omissions on some days, data for omissions were averaged across day for each phase (pre and post-surgery), resulting in three omission values per rat (one for each block of 50 trials) per phase (pre and post-surgery) - six values per rat in total.

Rats that received a cholinergic basal forebrain lesion made more omissions than sham operated rats (main effect of surgery: $F[1,10] = 21.35$, $p = 0.001$) and importantly there was a phase by group interaction ($F[1,10] = 20.72$, $p = 0.001$) i.e., the magnitude of the decrease in performance post operatively depended on whether the rats had received a lesion. As expected, rats made more omissions during the high event rate (middle) block of trials (main effect of block ($F[2,20] = 87.77$, $p < 0.005$)). Rats in both groups made more omissions after surgery (main effect of phase ($F[1,10] = 81.52$, $p < 0.005$)). Unlike for choice accuracy data, the interaction between phase and block for omissions did not quite reach significance ($F[2,20] = 3.34$, $p = 0.056$).

The interaction between phase and surgical group was decomposed by examining the effect of surgical group in each of the phases separately. Repeated measures ANOVAs were constructed for each of the phases (pre surgery and post-surgery) separately, with block (3 levels: first, middle and last block of 50 trials) as the within-subjects factor and surgical group (2 levels) as the between-subjects factor.

As expected there was a main effect of block ($F[2,20] = 13.1$, $p < 0.005$) on pre-surgical (baseline) omissions. Both sham operated rats and lesioned rats made a similar percentage of omissions (mean +/- sem sham: 3.73 +/- 0.85, lesion: 3.99 +/- 0.71; main effect of surgical group $F[1,10] = 0.055$, $p = 0.820$) during the pre-surgical

phase. There was no interaction between group and block ($F[2,20] = 0.045$, $p = 0.956$) for pre-surgical omissions (see Figure 4.11 for a representation of the average pre-surgical (baseline) values).

There was also the expected main effect of block ($F[2,20] = 47.83$, $p < 0.005$) on post- surgical omissions. Importantly, in the post-surgical phase lesioned rats made more omissions than sham rats (mean +/- sem sham: 8.86 +/- 1.56, lesion: 19.53 +/- 1.32; main effect of group $F[1,10] = 27.173$, $p < 0.005$). This effect of group on omissions also interacted with block ($F[2,20] = 4.88$, $p = 0.019$), reflecting that the difference between sham and lesion rats was greatest during the last block of trials (see Figure 4.11 for a representation of the average post-surgical values).

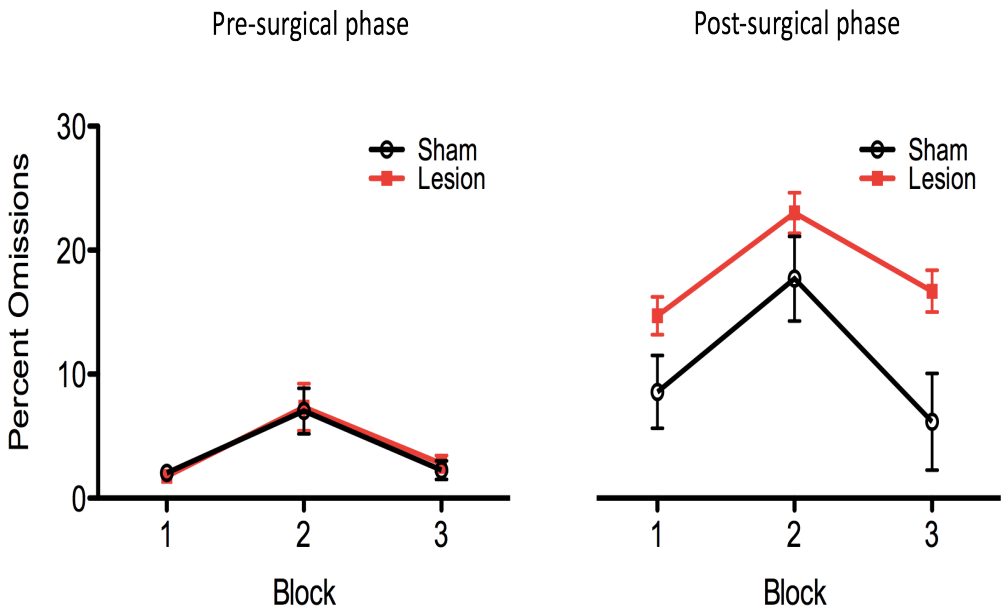

Figure 4.11 Percentage of omissions for sham operated rats and rats that received a cholinergic basal forebrain lesion, during the pre and post surgical phases. Data are presented as mean +/- sem for each block of 50 trials (blocks 1-3). Lesioned rats made more omissions, than sham operated rats, in the post-surgical phase.

Immunohistochemistry

Photomicrographs of ChAT immunostaining at the level of the injection sites in the basal forebrain are shown in Fig 4.12. Abundant ChAT positive (cholinergic) neurons are present in the brain of the control rat. A loss of ChAT-positive neurons is apparent in the brain of the rat that had received a lesion of the basal forebrain with a cholinergic specific neurotoxin. The loss of ChAT-positive neurons demonstrates (in addition to the expected result on post-lesion 5CSRTT performance) that lesion surgery was successful.

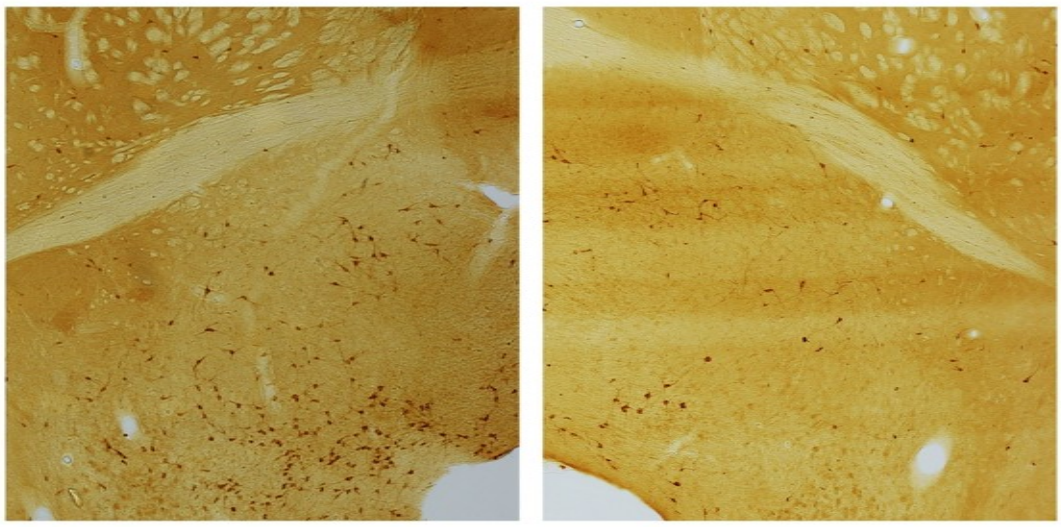

Figure 4.12 Representative photomicrographs of coronal ChAT stained brain sections at the level of the anterior commissure. Left hand image = a control rat. Right hand image = a rat that received injection of 192 IgG-saporin into the basal forebrain. Diminution of ChAT positive neurons (dark spots) can be seen in the right hand image.

Discussion

It was hypothesised that exposure to anaesthesia would lead to post-anaesthetic impairments in attention, and that the impairments would be greater in rats that had been repeatedly exposed to anaesthesia. Neither 2hrs of isoflurane plus nitrous oxide, or 2hrs of propofol plus fentanyl anaesthesia led to impairment in the sustained attention task (SAT). Mild anaesthetic-induced impairment was seen in the 5-choice serial reaction time task (5CSRRT), following 2hrs of isoflurane plus nitrous oxide anaesthesia. However, impairment was not seen in all performance measures, and was unexpectedly greatest after a single (not multiple) anaesthetic exposure.

The relative effects of anaesthesia on 5CSRTT and SAT performance

Performance of the 5CSRTT but not the SAT was impaired by 2hrs of isoflurane plus nitrous oxide anaesthesia. Both tasks are tasks of visual attention, and are known to be dependent on cholinergic function (Muir et al., 1994, Robbins et al., 1989), as was shown here in Experiments 1b (SAT) and 2c (5CSRTT). Unlike the SAT, it could be argued that the 5CSRTT contains a greater spatial component (Bari et al., 2008), with rats being required to monitor a horizontal array of response ports and make a response at the correct spatial location. Exposure to 2hrs of isoflurane plus nitrous oxide in adulthood impairs subsequent spatial memory performance on the radial arm maze (Chapter 3 and (Culley et al., 2004a, Culley et al., 2004b) (Culley et al., 2003b), it is therefore possible that the spatial component of the 5CSRTT is what renders task performance sensitive to the effects of anaesthesia.

There were two 5CSRTT performance parameters impaired by anaesthesia - the response time and the percentage of omitted trials. Food reward collection latency was similar between groups in the 5CSRTT, not only demonstrating that motivation to collect food reward was similar but also suggesting that the anaesthetic-induced impairment in response time was not due to a generalised motor impairment. The percentage of correct choices (accuracy) was also unaffected, perhaps suggesting that rats were only mildly impaired and able to 'trade off' omissions in order to maintain accuracy. It is of note that rats are less likely to omit trials when performing the SAT. Indeed, many rats made zero omissions on the SAT task. The visual stimulus is centrally located in the SAT operant chamber, with the food reward collection port on the same wall, underneath it. Unlike the 5CSRTT chamber (where the rat is required to turn away from the response ports to collect the food reward, and subsequently turn around and scan multiple ports), this set up enables the rat to monitor the stimulus port whilst collecting and eating the food reward, ensuring that an omission is less likely. It is proposed that the unequal sensitivity of the tasks for the omission parameter I.e., they just make more omissions in the 5CSRTT than the SAT, could account for the difference found between the two tasks.

Rats from Experiment 1 were tested during their light phase (housing room lights on at 0700h), whereas rats from Experiment 2 were tested during their dark phase (housing room lights on at 2000h). This unavoidable difference in husbandry conditions could conceivably be expected to interact with anaesthetic exposure and differentially affect task performance. However, as rats sleep during the light phase of the light cycle, anaesthetising and testing rats during this phase necessarily disturbs sleep and would therefore be expected to impair attention (Cordova et al., 2006). Given that attentional impairment was only seen in rats of Experiment 2, who were

tested during their dark phase (active phase), it seems highly unlikely that difference in this husbandry condition could account for the different results. In addition, unpublished data show that performance of the 5CSRTT is unaffected by the lighting phase in which rats are tested (McGaughy).

One of the striking features of the impairment in 5CSRTT performance is that it is a function of the change in event rate across blocks of trials. It is therefore most likely that an impairment in attention is only seen when the demands of the task are high and suggests that the demands of the SAT task were not high enough.

The relative effects of different anaesthetic regimens

Despite an impairment in 5CSRTT performance occurring following exposure to isoflurane plus nitrous oxide, an impairment was not found when rats were exposed to the same duration of propofol plus fentanyl anaesthesia. To the author's knowledge there are no other studies that have investigated the effect of anaesthesia on attentional function in animal models, with which to compare these results. However, agent specific impairments in post-operative cognitive function have been suggested from some human patient studies. In their study of 1987, Milligan et al used the four-choice reaction time task to test the post-operative cognitive function of patients anaesthetised for minor surgery, at three time points within the first hour of the recovery phase. The response time of patients anaesthetised with isoflurane plus nitrous oxide took longer to return to that of the control group, when compared to patients anaesthetised with *propofol* plus nitrous oxide (Milligan et al., 1987). In a more recent study, elective surgery patients tested with the digital symbol substitution test, a test of psychomotor

ability, were found to make less accurate choices up to 60 minutes after the end of surgery, if they had been anaesthetised with sevoflurane or desflurane (volatile anaesthetic agents similar to isoflurane) plus nitrous oxide rather than with propofol plus remifentanil (a short acting opioid drug) (Larsen et al., 2000). However, both of these studies investigated the return of cognitive function in the immediate recovery phase, rather than the longer term effects once the drugs have been eliminated from the body.

Rats anaesthetised with propofol plus fentanyl received a higher inspired oxygen concentration during anaesthesia and were more acidaemic at the end of anaesthesia, when compared to rats anaesthetised with isoflurane plus nitrous oxide. It is unlikely, however, that these differences in physiology could account for differences in task performance. Both propofol and fentanyl are known to depress respiratory function and this group therefore received a higher inspired oxygen concentration in order to compensate for this effect. The fact that there was no group difference in partial pressure of oxygen within the blood demonstrates that the compensation was effective and both groups had similar blood oxygen content levels. The lower blood pH (acidaemia) seen in rats that received propofol plus fentanyl was a consequence of the higher partial pressure of carbon dioxide within the blood (reflecting respiratory depression). This effect was an undesirable deviation from normal physiology in that group but was not associated with impairment in attention, and so does not seem to have had a consequence.

Evidence from animal models of persistent post-operative spatial memory dysfunction support the theory that the choice of anaesthetic agent maybe an important factor. As mentioned earlier, rats anaesthetised with isoflurane plus nitrous

oxide demonstrate post-anaesthetic impairment in spatial working memory when testing is begun 48hrs after cessation of anaesthesia (Culley et al., 2004b); an effect that persists in aged rats (Culley et al., 2004a), but not young rats (Crosby et al., 2005) if testing is begun 2 weeks after the cessation of anaesthesia. However, despite this apparent augmentation of anaesthetic-induced cognitive impairment with advanced age, older rats (18 mo) show no spatial memory impairment following 2hrs of propofol anaesthesia (Lee et al., 2008). The result of Experiment 2b is therefore consistent with the theory that propofol is devoid of the persistent effects on cognitive function observed with isoflurane plus nitrous oxide.

An alternative explanation for the differential effects of the anaesthetic regimens used here is that the regimens were not equipotent in terms of dose. When comparing the effect of two different anaesthetic regimens it is important that they are administered at equivalent doses. In this study the dose of propofol and fentanyl used was chosen based on experience, to most closely match the depth of anaesthesia (as assessed with the pedal withdrawal reflex) and physiological condition achieved with the isoflurane and nitrous oxide regimen used here. However, although the method of depth assessment (pedal withdrawal reflex response to toe pinch) is a widely accepted method, it is not precise; and so it is possible that rats that received propofol and fentanyl simply received a lower dose of anaesthesia than those that received isoflurane and nitrous oxide. Future use of a standardized stimulus (such as that used in non-human primate studies (Raper et al., 2015) would help to minimize this possibility.

The relative effects of single and multiple exposures to anaesthesia

Rats in Experiment 2a, exposed to isoflurane plus nitrous oxide, demonstrated a specific increase in omissions during conditions of high attentional demand, that was worse after the first exposure but not subsequent anaesthetic exposures. These results are unexpected when compared to that of Chapter 3 Experiment 2. In Chapter 3, rats that received repeated anaesthesia showed greater spatial working memory impairment than those that received a single exposure, implying that the detrimental effects of anaesthesia are cumulative. The lack of augmentation of the impairment in 5CSRTT with increased exposures could mean that the effects of factors affecting phenotypic expression could be specific to particular cognitive domains. Importantly however, unlike rats in the spatial working memory experiment, rats here were underwent behavioural testing after each episode of anaesthesia. It is therefore possible that the relative improvements in performance following the second and third periods of anaesthesia are confounded by a practice effect on the task. In order to determine whether rats become resistant to the effect of anaesthesia on attention, or increased practice with the task counteracts anaesthetic-induced impairment, a subsequent experiment could compare the performance of rats that receive one episode of anaesthesia 24 hours before testing begins, with those that receive repeated episodes of anaesthesia, the last of which occurs 24 hours before testing begins (the same design as that used in Chapter 3).

Conclusions

Taken together these analyses indicate that there is a mild effect of anaesthesia on attention that is specific to certain performance measures, as well as being agent specific and not augmented by repeated episodes of anaesthesia. Furthermore,

detection of the impairment is task dependent. Future work could examine the effect of factors that might exacerbate this mild phenotype, such as life stage.

CHAPTER 5: MULTIPLE, BUT NOT SINGLE, EXPOSURES TO ISOFLURANE DURING DEVELOPMENT IMPAIR WIN-SHIFT RADIAL ARM MAZE PERFORMANCE IN ADULTHOOD

Introduction

Exposure to anaesthetic agents early in development can cause neurotoxicity, and neonatal exposure in rodents is associated with persistent deficits in cognition in later adulthood, particularly in learning and memory (Hudson and Hemmings, 2011, Jevtovic-Todorovic, 2011). These deficits have been demonstrated following exposure to a variety of anaesthetic agents (Fredriksson et al., 2007, Jevtovic-Todorovic et al., 2003b, Satomoto et al., 2009) and are not restricted to agents of a particular drug class. Deficits occur following exposure at different perinatal developmental time points (Jevtovic-Todorovic et al., 2003b, Palanisamy et al., 2011, Zhu et al., 2010, Rothstein et al., 2008) although the most commonly demonstrated effect in rodents occurs following exposure at postnatal day (P) 7, a critical period of synaptic growth in brain development. Anaesthetic-induced cognitive deficits persist into adulthood and are thought to be dose and duration dependent. Unpublished meta-analysis of behavioural data from studies of developmental anaesthetic neurotoxicity finds that exposures of 6 hours plus are associated with cognitive impairment (Stewart, in prep).

Biomedical research procedures are carried out on neonatal animals for the purpose of investigating developmental biology and a wide range of diseases including, but not limited to, malnutrition (Puiman and Stoll, 2008), drug addiction (Richardson et al., 2006), oncological (Toth, 1968), infectious (Levast et al., 2013) and cardiovascular (Conway and Riley, 2011) disease. Advances in genetic modification (GM) technology facilitate the use and application of neonatal animal models, and the breeding of GM animals increased by 22% in the United Kingdom alone between 2011 and 2012 (Statistics, 2013). It is not known how many animals (neonatal or otherwise) are used in biomedical research in the United States, however it *is* known that the Food and Drug Administration agency recently outlined neonatal and paediatric drug development programs that will necessarily lead to an increase in the use of neonatal animal models (FDA, 2012). In short, the use of neonatal animal models is increasing as scientific and technological advances proceed. As with adult models, the identification and characterisation of factors (such as anaesthetic-induced cognitive impairment), that may confound the data resulting from experiments where they are used, are essential if interpretability of such data is to be maximised.

The mechanism by which anaesthetic-induced cognitive impairment occurs is currently unclear (Brambrink et al., 2012b). Exposure to anaesthesia causes widespread neuroapoptosis in the developing brain of a variety of species (Brambrink et al., 2012a, Istaphanous et al., 2011, Rizzi et al., 2008, Rizzi et al., 2010, Yon et al., 2005) and the greatest vulnerability appears to occur at the peak of synaptogenesis (Yon et al., 2005, Rizzi et al., 2010) implying that neuronal loss early in development leads to cognitive impairments later in life. There are, however, likely to be other mechanisms by which anaesthesia causes neural dysfunction and cognitive

impairment, including disturbances in the synaptic morphology of surviving neurons (Amrock et al., 2015, Briner et al., 2010, De Roo et al., 2009).

Repeated exposure of neonatal mice to sevoflurane has been shown to result in spatial memory deficits, when testing is conducted one month post-exposure (Shen et al., 2013). This study also reported that there were no effects of desflurane on cognitive performance, indicating that anaesthetic-induced cognitive impairment maybe agent selective. Repeated exposure of neonatal rats to isoflurane (a commonly used volatile anaesthetic agent and the one chosen for this study) has been shown to cause long-term cognitive deficits (Zhu et al., 2010), but the the effects of repeated exposures relative to that of a single exposure have not been studied. The extent to which cross-study comparisons can be made to answer this question is limited, as the conduct of behavioural experiments is inherently variable and disparity exists regarding whether a single dose of anaesthesia is, or is not (Shen et al., 2013, Loepke et al., 2009, Liang et al., 2010) sufficient to cause long-term cognitive impairment. The goal of the current study, therefore, was to directly compare the performance of adult rats, exposed to either single or repeated episodes of isoflurane anaesthesia as neonates, on the performance of a spatial memory task. This addresses the generality of whether multiple versus single exposures to anaesthetic agents during development have differential effects on long-term cognitive outcome.

Materials and Methods

Subjects

Postnatal day (P) 7 male or female Long Evans rat pups (Taconic USA), from 6 natural litters, were housed with their dams until weaning at three weeks of age, and then in same sex groups, in standard 'Individually Ventilated Cages', with automatically regulated lighting (12/12h light/dark cycle, lights on at 2000).

Anaesthesia

Episodes of anaesthesia consisted of 2 hours of 1.8% isoflurane delivered in 100% oxygen. Monitoring included chamber oxygen, carbon dioxide and isoflurane concentrations, and subject pulse oximetry (VitalStore, Vetronic Services Ltd, UK). Rectal temperature was monitored (PowerLab, ADInstruments Ltd, UK) and maintained at 36.5C +/- 0.5C. Rats in the control condition received 100% oxygen in identical environmental conditions to rats receiving anaesthesia. Anaesthetised rats were recovered in 100% oxygen for 20 minutes and returned to the dam with rats from the control condition (See Section 4, Chapter 2 - General Methods for detailed methods).

The starting point for this thesis was to build on work carried out by Culley et. al. (demonstrating anaesthetic-induced cognitive impairment after exposure to isoflurane and nitrous oxide during adulthood (REF Culley 2004) and Jevtovic-Todorovic et. al. (demonstrating anaesthetic-induced cognitive impairment after

exposure to isoflurane, nitrous oxide and midazolam during development (Jevtovic-Todorovic et al., 2003b). Which is why a combination of isoflurane and nitrous oxide was chosen for Chapters 3 and 4. However, as time passed the field became increasingly focused on single agents and moved away from investigations involving nitrous oxide. This shift in focus, in conjunction with discussion with colleagues in the field, dictated the change of anaesthetic regimen studied in this Chapter. It was considered important to study the effect of isoflurane alone in order to maintain generalisability with the rest of the field.

Experimental design

Male or female pups (P7) were allocated to one of four experimental groups, counter-balanced primarily for dam and, as far as was possible, for sex and then bodyweight. Rats either received a single 2-hr exposure to anaesthesia (1A: 5 males, 5 females) or control condition (1C: 7 males, 4 females) at P7 or, three 2-hr exposures to anaesthesia (3A: 6 males, 5 females) or control condition (3C: 6 males, 2 females) at P7, P10 and P13. At P91 rats underwent one trial, of spatial win-shift performance testing per day, for nine days.

In a separate experiment, rats that received 2hrs of 100% oxygen on P7 (control group, n = 8) and rats that received 2hrs of 1.8% isoflurane in 100% oxygen on (i) P7 (group P7: n = 8), (ii) P7 and P10 (group P10: n = 8) or, (iii) P7, P10 and P13 (group P13: n = 9) were used for mixed arterial/venous (cardiac puncture) blood gas analysis.

Samples were collected at the end of the period of anaesthesia (before recovery) and the pups immediately euthanased, after the last anaesthetic exposure. Control rats were exposed to 3% isoflurane in 100% oxygen until loss of righting reflex (approximately 20 seconds) just prior to blood sampling in order to prevent distress. Rats were removed from the anaesthetic chamber and a trans-cardial blood sample was immediately taken and analysed (Radiometer ABL80, Cleveland, USA) for pH, pCO_2 and pO_2.

Behavioural testing: win-shift radial arm maze

Spatial win-shift performance was tested on a 12-arm radial arm maze (RAM), where each of the 12 arms was baited and food rewards that were not replaced within trials (i.e., this is the same task as used in Chapter 3). This task is commonly referred to as a working memory task (see Chapter 3 for discussion). Rats from the four experimental groups were food-restricted from P81 and maintained at 85% of age matched ad libitum levels. At P88 rats were acclimated to the RAM for 5 minutes per day for 3 days. Beginning on P91 rats underwent one trial per day of win-shift RAM testing for nine days in order to evaluate performance of the task, (see Section 5.2 for detailed methods).

The following performance measures were recorded: the time to complete the maze (time required to obtain all 12 food rewards up to a maximum of 900 seconds); choices before the first error (number of arm choices made during a trial before the first error occurred) and omissions (number of unvisited arms at the end of a trial). A percent

correct measure was also calculated for the total number of choices made ((number of correct choices/total number of choices) x 100).

Statistical analysis

Data were analysed (SPSS v19) with independent sample t-tests (blood gases) or repeated measures analysis of variance (ANOVA) with day of testing (9 levels) as the within-subjects variable and group (3 levels - see second paragraph of behavioural testing results section for combination of control groups) and sex (2 levels) as between-subjects variables, with Fisher's LSD post hoc analysis (behavioural data). Results at the $p < 0.05$ level are considered statistically significant.

Results

Anaesthetic physiology

Body temperature was successfully maintained between 36C and 37C in all anaesthetised pups (those anaesthetised before behavioural testing and those anaesthetised before blood gas analysis). Blood oxygen saturation was successfully maintained above 95% in all anaesthetised pups (those anaesthetised before behavioural testing and those anaesthetised before blood gas analysis).

Blood gas values at each time point revealed moderate acidaemia in each of the three anaesthetic groups (P7: $p = 0.01$; P10: $p = 0.01$; P13: $p = 0.003$) compared to control rats (Table 5.1). Group P7 rats tended to have higher pCO_2 (hypercapnia) than control rats ($p = 0.09$). Hypercapnia relative to control rats was detected in groups P10 ($p =$

0.001) and P13 ($p = 0.05$) (Table 5.1). There was no difference in pO_2 between control and anaesthetised rats. There were no differences in pH, pCO_2 or pO_2 between groups that received anaesthesia.

	P7 control (n = 8)	**P7 (n = 8)**	**P10 (n = 8)**	**P13 (n= 9)**
pH	7.4 +/- 0.02	7.29 +/- 0.03*	7.25 +/- 0.05*	7.26 +/- 0.03**
pCO_2 (mmHg)	44.5 +/- 4.1	56.6 +/- 7.8	72 +/- 7.2**	59.6 +/- 5.8*
pO_2	196.5 +/- 34.2	191.8 +/- 35.2	209.1 +/- 50.5	212.6 +/- 52.1

*Table 5.1: Blood gas parameters for control rats who received 2 hours of 100% oxygen at P7; rats that received 2 hours of isoflurane anaesthesia at P7 (group P7), both P7 and P10 (group P10), or on P7, P10 and P13 (group P13). Rats that received anaesthesia were acidaemic compared to control rats. Rats in group P10 and group P13 were hypercapnic compared to control rats. * $p < 0.05$. ** $p < 0.01$.*

Behavioural testing: win-shift radial arm maze

All rats made at least 12 choices per trial. In terms of overall RAM performance, rats took less time to complete the RAM and made fewer errors and omissions across testing days. There was a main effect of day of testing for time to complete the RAM ($F(8,272) = 19.811$, $p < 0.0001$), choices before first error ($F(8,272) = 26.919$, $p < 0.0001$) and omissions ($F(8,272) = 21.178$, $p < 0.0001$). In other words, the performance of rats in each group improved across day of testing, demonstrating the absence of a ceiling effect[4] in any group.

Control group data (groups 1C and 3C) were compared and found not to be different for any of the RAM parameters. There was no main effect of group and no group by

[4] A ceiling effect is a measurement limitation that occurs when close to the highest possible score is reached throughout testing. Such an effect could reflect insensitivity of a task to detect differences between groups.

sex interaction for: time to complete the maze ($\underline{F}(1,15) = 0.176$, $p = 0.681$) ($\underline{F}(1,15) = 2.692$, $p = 0.122$); choices to first error ($\underline{F}(1,15) = 0.461$, $p = 0.507$) ($\underline{F}(1,15) = 4.347$, $p = 0.55$) or omissions ($\underline{F}(1,15) = 2.062$, $p = 0.172$) ($\underline{F}(1,15) = 0.0.213$, $p = 0.651$). The groups were therefore combined into a single control group (group C) for comparison with anaesthesia groups.

Time to complete the maze

Different measures of maze performance revealed different patterns of anaesthesia effects and suggested unexpected sex effects. For time to complete the maze there was a main effect of group ($F(2,34) = 4.633$, $p = 0.017$), an interaction between group and sex ($F(2,34) = 5.252$, $p = 0.01$) and between day of testing, group and sex ($F(16,272) = 2.71$, $p = 0.001$) (Fig 5.1). These interactions were decomposed with focused ANOVAs to specifically examine the 1A and 3A groups relative to controls. Comparison of groups C and 1A revealed interactions of group and sex ($p = 0.005$) and day, group, and sex ($p < 0.0005$). This interaction is driven by faster time to complete the maze of 1A males relative to male controls (main effect of group, $p = 0.02$, group x day interaction, $p = 0.001$) and by a slower time to complete the maze of 1A females relative to female controls, although this did not reach statistical significance (group x day interaction, $p = 0.074$). This lack of statistical significance for the female comparison suggests that the study may be underpowered and therefore that these results should be interpreted with caution (see Discussion). 1A males outperformed 1A females (sex x day interaction for 1A rats only, $p = 0.049$). Female controls outperformed male controls (sex x day interaction for C rats only, $p = 0.002$, main effect of sex $p = 0.033$). Parallel analyses comparing groups C and 3A revealed a main effect of group ($p = 0.014$) that did not interact with sex ($p = 0.224$). There was

a trend towards an interaction of group (C vs 3A) with sex and day ($p = 0.058$), again suggesting that power may be an issue for this study.

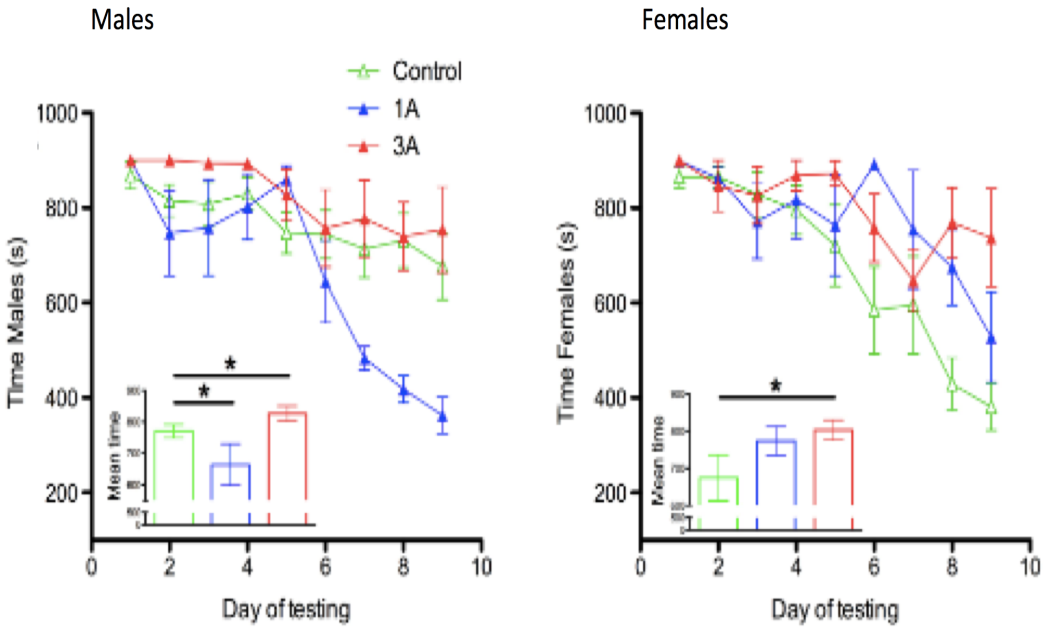

*Figure 5.1: The time taken to complete the maze task, up to a maximum of 900 seconds. Rats exposed to multiple (but not a single) episodes of anaesthesia took longer to complete the maze than control rats. Rats were exposed to 2 hours of isoflurane plus nitrous oxide anaesthesia at postnatal day P 7 (blue), or at P7, P10 and P13. Control rats were exposed to 100% oxygen (green). Data are represented as mean +/- sem. *p < 0.05.*

Number of choices before the first error occurred

Male rats demonstrated an unexpected increase (compared to controls) in the number of choices made before the first error occurred, following exposure to a single episode of anaesthesia. For choices to first error (Fig 5.2) there was a main effect of group ($F(2,34) = 5.249$, $p = 0.01$) and an interaction between sex and group ($F(2,34) = 3.548$, $p = 0.04$). Decomposition of these effects revealed a similar pattern to time, where this interaction was driven by facilitated performance of 1A males relative to male controls. The performance of 3A rats compared to controls was numerically poorer ($p = 0.098$), which was driven entirely by the females (females: $p = 0.027$; males: $p = 0.743$).

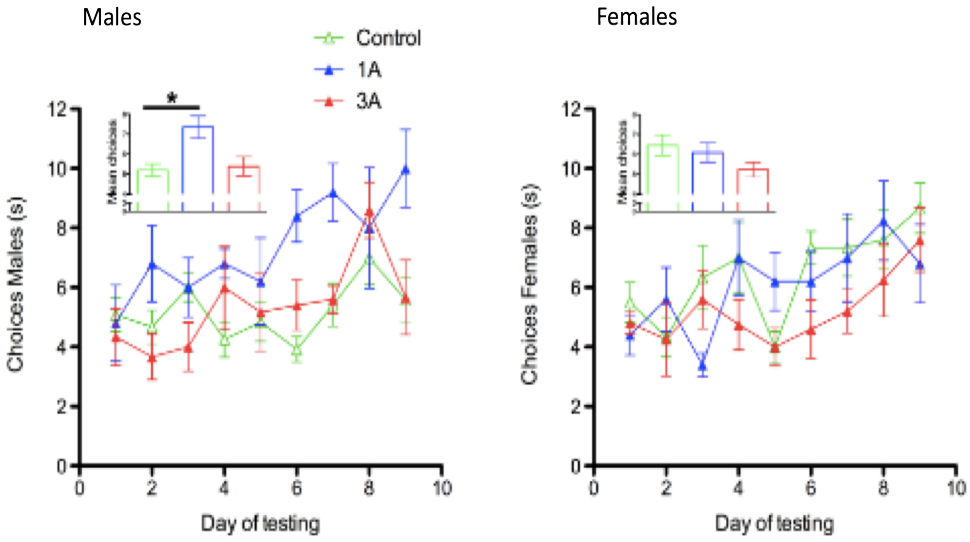

*Figure 5.2: The number of choices made before the first error occurred. Male rats (left) exposed to a single episode of anaesthesia made more correct choices before the first error was made, than control rats. Rats were exposed to 2 hours of isoflurane plus nitrous oxide anaesthesia at postnatal day P 7 (blue), or at P7, P10 and P13. Control rats were exposed to 100% oxygen (green). Data are represented as mean +/- sem. *p < 0.05.*

Number of omissions

Multiple exposures to anaesthesia were associated with a greater number of omissions. There was a main effect of group for omissions ($F(2,34) = 3.791$, $p = 0.033$) (Fig 5.3) that did not interact with sex or day. Group 1A did not differ significantly from group C on this measure ($p = 0.602$) but group 3A did ($p = 0.042$). These effects also did not interact with sex.

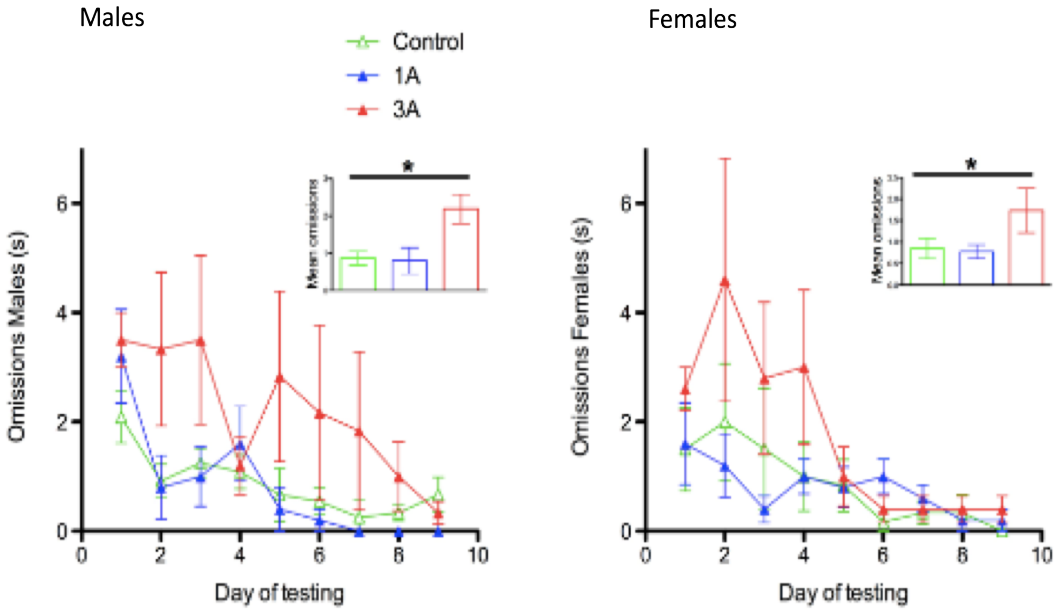

*Figure 5.3: The number of omitted (unvisited) arms within a trial. Rats exposed to multiple (but not a single) episodes of anaesthesia made more omissions than control rats. Rats were exposed to 2 hours of isoflurane plus nitrous oxide anaesthesia at postnatal day P 7 (blue), or at P7, P10 and P13. Control rats were exposed to 100% oxygen (green). Data are represented as mean +/- sem. *p < 0.05.*

Percentage of correct choices

The percentage of choices made that were correct (percent correct) was analysed, and found to be greater in male rats following a single anaesthetic exposure and lower in female rats following multiple exposures. There was a main effect of day of testing for percent correct ($F(8,272) = 4.802$, $p < 0.0001$), representing the expected improvement in performance across day of testing. There was no main effect of group for percent correct ($F(2,34) = 2.069$, $p = 0.142$) however, there were interactions between group and sex ($F(2,34) = 5.689$, $p = 0.007$), and between day of testing, group and sex ($F(16,272) = 2.215$, $p = 0.005$) (Fig. 5.4). Decomposition of these effects revealed that interactions were again driven by facilitated performance of 1A males relative to male controls ($p = 0.003$), and impaired performance of 3A females relative to female controls ($p = 0.031$).

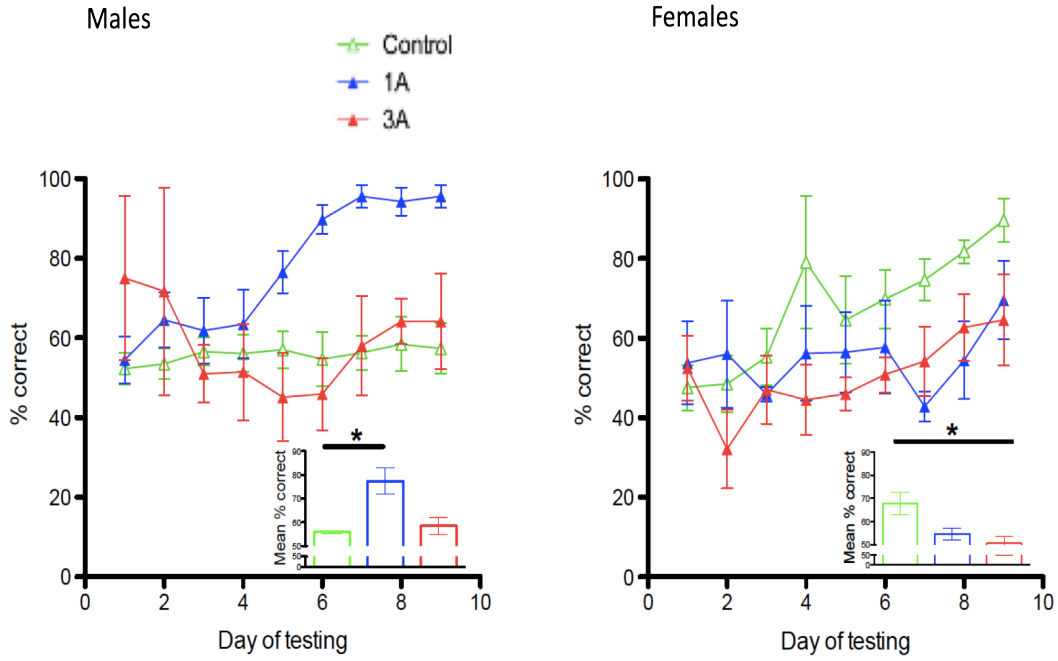

*Figure 5.4: The percentage of choices made that were correct. Female (right) but not male (left) rats exposed to multiple episodes of anaesthesia, made a lower percentage of correct choices, within a trial, on a win-shift radial arm maze task. Rats were exposed to 2 hours of isoflurane plus nitrous oxide anaesthesia at postnatal day P 7 (blue), or at P7, P10 and P13. Control rats were exposed to 100% oxygen (green). Data are represented as mean +/- sem. *p < 0.05.*

Discussion

Overall, these results indicate that repeated exposure to anaesthesia but not a single exposure, early in development, may produce persistent spatial memory impairment in female rats when tested as adults. However, although repeated exposure to anaesthesia was associated with impairments in male rats when tested as adults, a single exposure appeared to, paradoxically, improve performance. These effects are seen against a background of sex differences in control animals on these measures, with females tending to perform better than males.

Repeat episodes of anaesthesia were more detrimental to win-shift performance than a single episode

The principal finding of this study is that repeated exposures to isoflurane during development are associated with greater long-term impairment, in the ability of adult rats to perform a spatial memory task, than a single exposure. Rats of both sexes that had been repeatedly exposed were slower to complete the maze and made more errors of omission (failure to visit baited arms within the time allowed for maze completion). These results suggest that anaesthetic exposed rats are cognitively impaired. However, it should be noted that these two parameters may be affected by psychological or sensory-motor functions - such as running speed or motivation - not tested here. The percentage of correct choices made is more likely to reflect memory function *per se* and on this measure only female rats were impaired.

Single exposure to isoflurane revealed an unexpected apparent facilitatory effect in males, with single-exposed males completing the maze faster than male controls, and

making more choices before the first error occurred. Collectively these findings support the view that repeated exposures to general anaesthesia during development are associated with greater long-term cognitive impairment than a single exposure. Furthermore, although not the focus of this study, they raise the possibility that sex may be a critical variable in the neuro-developmental outcomes of early-life anaesthetic exposure.

Population-based, retrospective birth cohort studies demonstrate that children who receive multiple (but not a single) general anaesthetics at a young age are at increased risk for learning disability and attention deficit disorder (Wilder et al., 2009, Sprung et al., 2009, Sprung et al., 2012, Flick et al., 2011). This remains the case when results are adjusted for co-morbidities (Flick et al., 2011, Sprung et al., 2012). Results here are consistent with these studies, by demonstrating a greater detrimental effect on cognition of multiple exposures of general anaesthesia relative to a single anaesthetic exposure, in the absence of surgery and other associated environmental factors that cannot be controlled for in patient studies.

Repeated exposure of neonatal rats to isoflurane has previously been shown to cause long-term cognitive deficits. Zhu and colleagues demonstrated that adult rats, that received 35 minutes of isoflurane anaesthesia daily, for five consecutive days, from P14, were impaired on an object recognition task in comparison to control rats (Zhu et al., 2010). Our study further demonstrates that the magnitude of the deficits resulting from repeated exposures to isoflurane early in life, is greater than those resulting from a single exposure, and occurs in a different cognitive domain to that demonstrate by Zhu and colleagues.

Possible mechanisms for anaesthetic-induced impairment in win-shift RAM performance

Anaesthetic-induced neuroapoptosis in the developing brain has been shown to be age-dependent, at least in rats. The greatest vulnerability in rats occurs at P7, with little or no increase in apoptosis at P10 or P14 (Yon et al., 2005). If neurocognitive impairment following anaesthetic exposure is a direct result of anaesthetic-induced neuroapoptosis, repeated exposure may be associated with either cumulative neuron loss, sensitisation to the effects of subsequent exposures, or a combination of these factors. In particular, these results could be attributed to greater cumulative exposure in the repeat exposure group (a total of 6 hours) relative to the single exposure group (2 hours). But because the subsequent exposures occurred on days at which single exposures would not be expected to produce marked neuroapoptosis on their own (P10 and P13), these findings suggest that a more complex mechanism may be at work. Persistent alterations in the ultrastructure of surviving neurons also occur following anaesthetic exposure. These have been shown in rats to differ depending on the developmental stage that exposure occurs; with decreases in dendritic spine density occurring at P5 and P10 (Briner et al., 2011) and increases occurring at P15, P20 and P30 (De Roo et al., 2009). On this view, the deficits in neurocognitive function in the group that received multiple exposures here, might be the result of apoptotic neurodegeneration on P7 combined with alterations in spine density on P10 or P13.

The mechanism of anaesthetic-induced decreases in dendritic spine density, during development, are further supported by studies demonstrating that isoflurane leads to a decrease synaptic density. In addition to this effect, the administration of isoflurane

to cultured hippocampal neurons causes activation of the growth factor receptor p75. When isoflurane is administered in conjunction with an inhibitor of the p75 receptor the effects of isoflurane on synaptogenesis are reduced; supporting a role for the growth factor receptor in isofluranes effect on axonal transport and signaling (Head et al., 2009).

Developmental exposure of rodents to isoflurane has been shown to induce a persistent and progressive impairment in object recognition (a behavioural task not dissimilar to the Spontaneous Spatial Novelty Preference task used here in Chapter 3). Cognitive impairment was paralleled by a decrease in hippocampal stem cells in the absence of apoptosis. This decrease in neurogenesis was seen when isoflurane was administered during development and not when administered during adulthood, suggesting that the mechanisms underlying anaesthetic-induced cognitive impairment differ depending on the stage of life that the anaesthetic agent is administered (REF Zhu et al., 2010).

Mechanisms unrelated to neuronal structure, genesis or death have also been proposed for anaesthetic-induced cognitive impairment. Glial cells are the most abundant cells in the nervous system, providing support and insulation to neurons and playing a critical role in hippocampal synaptic transmission (Keyser and Pellmar 1994). Invitro studies have shown that isoflurane impairs glial cytoarchitecture in immature astroglia (Lunardi et al., 2011; Culley et al., 2013).

Understanding the mechanism by which multiple anaesthetic exposure, causes long-term cognitive impairment, and may be cumulative or involve sensitization to further effects of anaesthetic exposure, is a critical topic for future research.

The effects of a single exposure to anaesthesia appeared to differ depending on the sex of the subjects

Single exposure to anaesthesia indicated an unexpected sex effect, with single-exposed males performing better than male controls and single-exposed females tending to perform worse than female controls, and significantly worse than single-exposed males. As sex differences were not the focus of this study, and the numbers of rats of each sex is relatively low, the study may not be powered adequately to avoid a type I error (false positive), these results should be interpreted with caution but may warrant further study.

Sex differences in the spatial memory performance of normal (control) subjects have long been observed in many species, including humans (Eals, 1994) and rats (Roof, 1993), with the direction of the sex difference depending on the design of the behavioural task (Postma et al., 2004). Here, control females tended to perform better than control males, and this effect reversed in single-exposed rats. A reversal of the direction of sex difference in RAM performance occurs in rats that undergo chronic stress prior to behavioural testing (Bowman et al., 2003). It could be argued therefore that the control condition (2 hours of maternal separation) in this experiment provided a stressor. The resulting reversal of the sex effect in control animals would lead to an apparent but false improvement in the performance of once-exposed males relative to controls. This explanation seems unlikely, given that the stress paradigms used to demonstrate this effect involve a chronic stressor administered over days rather than hours. However, future experiments could be designed to exclude this possibility by only briefly separating control animals from the dam.

Sex differences in rodent post-anaesthetic cognitive impairment have been demonstrated. Shen and colleagues found that neonatal male mice exposed to a single episode of sevoflurane anaesthesia had facilitated escape latency performance on the Morris water maze (Shen et al., 2013). In addition, female but not male rats exposed to a single episode of anaesthesia (with isoflurane, nitrous oxide and midazolam) at P7, showed impaired acquisition of place trial learning in the Morris Water Maze (Boscolo et al., 2013b). Rothstein and colleagues found that male rats exposed to isoflurane anaesthesia at P0 demonstrate poorer performance on a reference memory radial arm maze task, than females (Rothstein et al., 2008). All of the above tasks assess spatial memory, although there are of course variations in study design, such as the precise testing methodology or time point that testing occurs at, that could account for differences.

One explanation for the apparent improvement in performance of single exposed male rats is that the difference actually reflects poor performance of male controls, not improved performance of anaesthetised males. Male controls performed more poorly than female controls, and remained at around chance performance (4 choices before the first error occurred) for much of the testing period (Fig 5.2). An improvement across testing days is not a necessary feature of win-shift performance, however, this behaviour contrasts that of female rats (and male rats from Chapter 3) and so should perhaps be considered when interpreting these data.

As mentioned previously, potential mechanisms for post-anaesthetic cognitive impairment in neonates include anaesthetic-induced alterations in neuronal ultrastructure. To date, such effects of neonatal exposure to anaesthesia have only been studied in males but sex is known to interact with other physiological stressors

that lead to alterations in rodent neuronal ultrastructure. Hippocampal CA1 dendritic spine density in male versus female rats responds in the opposite direction to the same physiological stimulus (Shors et al., 2001) and alterations of prefrontal cortex neuronal structure in the offspring of dams that undergo gestational stress, occur in a sex-specific manner (Murmu et al., 2006). On this basis it could be that sex may interact with the effect of anaesthesia on dendritic spine morphology (as do other physiological stressors) resulting in sex-dependent effects on long-term cognitive outcome. This provides a potential mechanism by which sex could affect the developmental time course of post-anaesthetic cognitive impairment. Further studies specifically designed to investigate behavioural sex differences are required, to fully characterise and understand the importance of these incidental observations.

As mentioned earlier, an important limitation to this study is the relatively low number of control females (n = 6) used. This low sample size may mean that the study is in fact underpowered to avoid a statistical type I error, these results should therefore be interpreted with caution. The above discussion of possible mechanisms to explain the sex effect found here is provided in support of further study, rather than as an explanation of this effect.

Could sex hormones have influenced the study outcome?

In order to avoid the potential confound of gonadectomy (McCarthy, 2005), intact female rats were used, and so any sex effect is potentially confounded by hormonal variation. In addition, subjects were tested at exactly the same developmental time point rather than at a particular stage of oestrus. However, it is of note that the

increase in variability that these factors introduce would not increase the likelihood that an effect would be detected between females in the three anaesthetic groups.

Anaesthetised rats demonstrated alterations in respiratory physiology during anaesthesia

Hypercapnia and acidaemia were observed in anaesthetised rats at each time point in this study. Whilst this demonstrates anaesthetic-induced physiological impairment, it is highly unlikely that this could have resulted in the behavioural differences demonstrated. Equivalent levels of hypercapnia and acidaemia were seen in rats that received a single exposure to anaesthesia and multiple exposures to anaesthesia. For this reason it seems unlikely that the spatial memory deficits seen in the multiple anaesthetic exposure group, as compared to the single exposure group, are due to respiratory disturbances. Additionally, it has been demonstrated that rats exposed to 4hrs of carbon dioxide at P7 do not develop long-term spatial memory deficits (Stratmann et al., 2009), showing that hypercapnia alone is not a sufficient causal factor.

Rats in this study demonstrated a mixed arterial/venous partial pressure of oxygen of approximately 200 mmHg. Rats were exposed to 100% oxygen as the carrier gas and would therefore be expected to demonstrate partial pressures of oxygen approximately three times higher. The lower values however, most likely reflect the fact that rat pups were removed from the anaesthetic chamber for blood gas sampling, were breathing room air containing 21% oxygen rather than 100% oxygen at the time of sampling, and are not therefore indicative of pulmonary disease. It is of note that the blood oxygen saturation levels were maintained above 95% for all rats. Delivery of anaesthetic (and

oxygen) via a mask would however eliminate this potential confound for future studies.

Were the development time points for anaesthetic exposure of relevance to human health?

The developmental time point chosen for first exposure to anaesthesia was postnatal day 7. This developmental time point is commonly used to investigate neonatal anaesthetic neurotoxicity; in part because the peak of synaptogenesis occurs around this age in rats (Dobbing and Sands, 1979) and is thought to be related to the period of greatest vulnerability. The correspondence of this age to a specific neurodevelopmental stage in humans depends on which measures are used to make the comparison. There are, however, inherent difficulties with making such comparisons. According to morphological measures, postnatal day 2-7 in rats corresponds to the human third trimester, and the brain growth spurt occurring at birth in humans is centred around P7 in rats (Clancy et al., 2007a). However, according to a neuroinformatics model (Clancy et al., 2007b), which integrates a number of different measures into a computational model, the stage of development of the brain in humans at birth is equivalent to P10 in rats. It could be argued that this study models multiple anaesthetic exposures beginning at a stage (late third trimester), during which anaesthesia is rarely needed. The stage of pregnancy, however, for parturition of surviving premature human infants is decreasing as advances in critical care progress. Infants born as early as the middle of the second trimester may now survive (Weber et al., 2005, Stoll et al., 2010). Many of these infants receive sedation and anaesthesia as part of their care. The choice of P7 as a

stage of anaesthetic exposure in rats, therefore represents an increasingly clinically relevant experimental model, regardless of the precise correspondence to stages of human brain development.

Summary

The immediate implication of these results is that a single short (2-hour) exposure to isoflurane produces little or no long-term cognitive impairment relative to multiple short exposures; and that multiple short exposures are sufficient to cause long-lasting (three months post-exposure) cognitive impairment in rats. Furthermore, the long-term cognitive effects of early anaesthetic exposure may differ between the sexes, suggesting sex as a candidate for investigation in future studies of developmental anaesthetic neurotoxicity, to confirm and extend or refute these results.

CHAPTER 6: EFFECT OF EXPOSURE TO ANAESTHESIA DURING DEVELOPMENT ON ATTENTION

Introduction

Developmental exposure to both alcohol and general anaesthetic agents have been shown to trigger apoptotic neurodegeneration in animal models (Ikonomidou et al., 2000, Jevtovic-Todorovic et al., 2003b), and this has been proposed as a mechanism for the resulting behavioural impairments. Anaesthetic-induced neurodegeneration occurs throughout cortical and subcortical areas, including anatomical regions that are involved in attentional processing (Jevtovic-Todorovic et al., 2003b). However, although evidence from animal experiments for anaesthetic-induced impairments in learning and memory is compelling (Jevtovic-Todorovic et al., 2013), there is currently no evidence regarding the presence or absence of post-anaesthetic attentional impairments.

In order to investigate whether anaesthetic exposure during development would lead to post-anaesthetic impairments in attentional processing, the effect of exposure to sevoflurane was tested in two behavioural tasks that are commonly used to assess attention in rodents. The most commonly used volatile anaesthetic agent used for laboratory animal anaesthesia is isoflurane. However, induction of anaesthesia with isoflurane is aversive (Moody and Weary, 2014), a property hypothesised to be due to its mucosal irritant properties. Because sevoflurane causes less irritation of mucosa (Kichko et al., 2015) it is gaining in popularity as an agent for laboratory animal

anaesthesia. Recent rodent studies have associated sevoflurane during development with alterations in socio-emotional behaviour (Satomoto et al., 2009), impairments in spatial reference memory and neuroapoptosis (Istaphanous et al., 2011, Zheng et al., 2013) as well as dose-related damage to hippocampal neuronal ultrastructure (Amrock et al., 2015). It is not known how general the effects of sevoflurane exposure are and thus far no data exist on the effects of sevoflurane on attentional processing.

The two behavioural tasks chosen for this study are, the sustained attention task (SAT) as devised by Bushnell and colleagues in 1994, validated by (McGaughy and Sarter, 1995) and used in Chapter 4 here; and the intra-dimensional extra-dimensional set shift task (IDED). A number of manipulations were made to the SAT, in order to alter the attentional demands of the task. The allocation of attentional resources may be controlled by 'top down', and 'bottom up' processes (Sarter et al., 2001). This hypothetical categorisation of processes distinguishes between knowledge driven mechanisms (top down), that facilitate the discrimination between relevant and irrelevant stimuli for instance, and mechanisms of signal detection (bottom up). By altering the parameters of the SAT task, it is possible to introduce task irrelevant stimuli that either provide distraction or facilitate signal detection (alter top down processing of stimuli), and to manipulate characteristics of the stimuli or the context in which they are presented (alter bottom up processing of stimuli) (Newman and McGaughy, 2008). By testing rats on a number of different task manipulations, the aim was to determine if rats that had been exposed to anaesthesia during development were less able to engage either top down or bottom up attentional processing.

The IDED task is based on the Wisconsin Card Sorting task (WCST) used to assess attentional set shifting, or 'flexibility of thinking' in humans (Berg, 1948, Milner,

1963). In the WCST stimulus cards are presented to a participant and differ in the type, number and colour of the symbols represented on them. The participant is not told how to match the cards but *is* told when they match correctly, enabling to participant to acquire the correct rule of classification (or response set) to attend to. Throughout the task the classification rules are changed without notice, requiring the participant to learn (or shift to) a new response set. The task thus assesses the ability of a subject to maintain a type of behavioural response when contingencies are unchanged, and alter the type of behavioural response in the face of changing contingencies. Performance of the task is dependent on the function of the prefrontal cortex, as well as recruitment of other brain areas depending on the stage of the task (Milner, 1963, Monchi et al., 2001).

In the rat version of the task, subjects are trained to dig for food reward, whose location is associated with a specific exemplar of a particular stimulus dimension (e.g., odour within the digging pot) of a compound stimulus consisting of three dimensions (the odour and digging media within the digging pot and the texture covering the digging pot - equivalent to the type, number and colour of the symbols used in the WCST). During the intra-dimensional shift phase of the task, rats are required to respond to (shift attention to) new exemplars of the same dimension (e.g., patchouli odour rather than cinnamon). Subsequently, during the extra-dimensional shift phase rats are required to respond to a new exemplar of a different dimension (e.g, digging media rather than odour), and therefore shift attention to a different 'set' of stimuli. Perseveration to a particular exemplar can also be measured, during reversal phases of the task when rats are required to respond to previously incorrect stimuli. The requirement to respond to different stimulus dimensions in the extra-dimensional shift phase of the task mirrors the requirement to shift response sets (for example, from

shape to colour) in the human WCST (Birrell and Brown, 2000). As in humans, the ability of rats to mediate attentional set shifting is dependent on the function of the frontal cortex (Birrell and Brown, 2000). Neurochemical specific lesion studies have demonstrated that rats with noradrenergic, and not cholinergic, depletion of the medial frontal cortex are impaired at the task (McGaughy et al., 2008) (Newman et al., 2008).

The effect of single versus repeated anaesthetic exposure during development, in males and females, was investigated in these experiments. It was hypothesised that exposure to anaesthesia during development would lead to impairments in attention in adulthood, and that the impairments would be greater in rats that had been repeatedly exposed to anaesthesia.

Materials and Methods

Subjects

Male or female Long Evans rat pups (Taconic USA), from 12 natural litters, were weaned at three weeks of age, and then housed in same sex groups, in standard 'Individually Ventilated Cages', with automatically regulated lighting (12/12h light/dark cycle, lights on at 2000). Transfer of animals to a different Institution was required for behavioural testing and here rats were kept on a 12/12h light/dark cycle with lights *off* at 1900h.

Anaesthesia or control conditions

Episodes of anaesthesia consisted of 2 hours of 2.5% sevoflurane delivered in 30% oxygen. Monitoring included chamber oxygen, carbon dioxide and isoflurane concentrations, and subject pulse oximetry (VitalStore, Vetronic Services Ltd, UK). Rectal temperature was monitored (PowerLab, ADInstruments Ltd, UK) and maintained at 36.5C +/- 0.5C. Rats in the control condition received 30% oxygen in identical environmental conditions to rats receiving anaesthesia. Anaesthetised rats were recovered in 30% oxygen for 20 minutes and returned to the dam with rats from the control condition (see Section 4, Chapter 2 - General Methods for details).

Male or female pups (P7) were allocated to one of three experimental groups, matched primarily for dam and sex and then, as far as possible, bodyweight. Rats received either a single 2-hr exposure to anaesthesia (group 1A: 5 males, 5 females) at P7; three

2-hr exposures to anaesthesia (group 3A: 5 males, 5 females) at P7, P10 and P13; or three 2-hr exposures to the control condition (group C: 5 males, 5 females). At P21 rats were weaned and subsequently pair housed. Behavioural testing, which was carried out during adulthood, included the sustained attention task (Experiment 1a) and attentional set shifting (Experiment 1b). All rats underwent sustained attention task testing first (at approximately P91), followed by attentional set shifting (at approximately P120).

In a separate experiment rats that had received, 2hrs of 2.5% sevoflurane in 30% oxygen on (i) P7 (group P7: n = 4), (ii) P7, P10 and P13 (group P13: n = 4) or, 30% oxygen without anaesthesia on (iii) P7, P10 and P13 (group C: n = 4) were used for mixed arterial/venous blood gas analysis. Samples were collected at the end of the period of anaesthesia (before recovery) and the pups immediately euthanased. Rats were removed from the anaesthetic chamber and a trans-cardial blood sample was immediately taken and analysed (Radiometer ABL80, Cleveland, USA) for pH, pCO_2 and pO_2. Control rats in group C, although not in an anaesthesia group, were exposed to 6% sevoflurane in 30% oxygen until loss of righting reflex (approximately 20 seconds) just prior to blood sampling, in order to prevent the distress associated with the procedure.

Experimental design

Experiment 1: Sustained attention task

Table 6.1 shows a representation of the chronology of the experimental phases. Behavioural task training began at approximately P75, to ensure that testing took place at approximately P91 (as in Chapter 5: Effect of single and repeated episodes of anaesthesia, during development, on spatial working memory). Rats were food restricted from two weeks before training began (see Section 3.3, Chapter 2 - General Methods for details) and underwent training on the task to a pre-determined criterion on the final shaping schedule (see Section 5.3, Chapter 2 - General Methods for details). All rats reached criterion. Baseline data consisted of data from the second of two consecutive daily sessions, where criterion was reached on the basic task version (which was the same as the final shaping schedule). A session consisted of a maximum of 162 consecutive trials or approximately 40 minutes per day. Rats were tested on a series of different task manipulations aimed at altering the attentional demands of the task. Between the different task versions, rats were re-trained to baseline performance and again, these baseline data consisted of data from the second of two consecutive daily sessions where criterion was reached on the basic task version.

Order	Experimental design phase	Comments
1	Group allocation	On postnatal day 7 - matched for sex, counterbalanced for dam and as far as possible for bodyweight
2	Anaesthesia	From postnatal day 7 − 3 groups: Control condition (group C), single exposure to sevoflurane (group 1A), repeated exposures to sevoflurane (group 3A)
3	Food restriction	From 2 weeks before training began - approximately postnatal day 77
4	Task training	From postnatal day 91 - To criterion of >75% hits to 500ms signals and >75% correct rejections to non-signal trials
5	Baseline testing	1 day of testing (basic task version)
6	Testing on task manipulation 1	1 day of testing
7	Re-baseline	To criterion on basic task version for two days: data = second of those days
8	Testing on task manipulation 2	1 day of testing
9	Re-baseline	To criterion on basic task version for two days: data = second of those days
10	Testing on task manipulation 3	1 day of testing
11	Re-baseline	To criterion on basic task version for two days: data = second of those days
12	Testing on task manipulation 4	1 day of testing
13	Re-baseline	To criterion on basic task version for two days: data = second of those days
14	Testing on task manipulation 5	1 day of testing

Table 6.1 Chronology of experimental phases for Experiment 1 - effect of exposure to anaesthesia during development on performance of the sustained attention task. Task manipulations are as follows 1 = high event rate session, 2 = short duration stimulus session, 3 = predictable tone, 4 = predictable light distractor, 5 = irregular light distractor. The order in which rats carried out the task variations was counterbalanced, as far as possible, for group and sex using a within subjects, latin square design.

Experiment 2: Attentional set shifting

Experiment 2 was carried out with the same cohort of rats as Experiment 1. Rats underwent behavioural task training and testing as they finished Experiment 1 (i.e., training and testing were staggered) and rats remained food restricted until completion of the intra-dimensional / extra-dimensional set shifting (IDED) testing.

Unfortunately, 4 rats took so long to complete the sustained attention task training and testing that they were unable to enter Experiment 2. In addition, one rat that was trained for Experiment 2 failed to make choices during the testing phases and was excluded from the study. The final experimental numbers were therefore as follows, group C: 5 females, 4 males; group 1A: 5 females, 4 males; group 3A: 3 females, 4 males.

This experiment took three days for each rat to complete. Behavioural task training began at approximately P120 and consisted of two days of training to dig for food reward (shaping) (day 1) and exemplar training (day 2) (see below for details). Testing on the IDED task took place on day 3 and consisted of a series of seven stimulus discrimination tasks (phases), described in detail below. Training and testing was conducted between 9am and 2pm.

Behavioural testing

Experiment 1: Sustained attention task

Please refer to Section 5.3, Chapter 2 - General Methods, for details of task training. The same apparatus, task training and baseline task protocol were used as in Chapter

4, for details of task manipulations skip to task variation details below (third paragraph). Sustained attention performance was tested in operant chambers where rats were food rewarded for distinguishing between signal (visual stimulus) and non-signal (absence of visual stimulus) trials by pressing the appropriate one of two levers extended after presentation (or lack of presentation) of the stimulus. Signal duration varied, such that either 500, 100 or 25 ms signals were presented in a pseudo-random (unpredictable) order. Following signal presentation or absence of presentation there was a short delay before the levers were extended. Each trial was separated by a variable (unpredictable) inter-trial interval (12 +/- 3 s). Animals underwent one session of up to 162 trials per day within 40 minutes.

Animals were trained in sound attenuated operant chambers (Med Associates, St. Albans, VT) equipped with two retractable levers, a house light, a pellet dispenser, a sonalert tone generator, and three panel lights. Incorrect lever presses were defined as misses when they occurred on a signal trial and false alarms when they occurred on a non-signal trial. If the animal failed to respond or responded incorrectly, the levers were retracted and the inter-trial interval (ITI) (12 +/- 3s) was reinstated. Signal duration varied and all trials were presented in a temporally unpredictable fashion to heighten attentional demands. Rats performed one session per day and were trained to a criterion of >75% hits to 500ms signals and >75% correct rejections to non-signal trials for a maximum of 162 consecutive trials or 40 minutes, for at least two consecutive sessions. After task training was complete, baseline testing consisted of one day of testing on the final shaping schedule (see Section 5.3, Chapter 2 - General Methods for details), 24hrs before being tested, for one session, on the first of the five task manipulations. Between task manipulations rats were re-trained to baseline performance on the basic task. The order in which rats carried out the task variations

was counterbalanced, as far as possible, for group and sex using a within subjects, latin square design.

The five task variations were as follows:

High event rate

The inter-trial interval was decreased from 12s +/- 3s to 9s +/- 3s for all trials within the session. A higher event rate was expected to increase the attentional demands of the task, in a similar fashion to that of the high event rate manipulation of the 5-choice serial reaction time task used in Chapter 4: "The effect of exposure to anaesthesia during adulthood on attention". Rats performing the high event rate SAT demonstrate a decreased ability to detect signals as the session progresses (McGaughy and Sarter, 1995). It was therefore predicted that the high event rate session would represent increased attentional demand.

Short duration stimulus

In this task variation presented stimuli were all 25 ms duration (i.e., the session contained 81 trials of 25 ms signal duration, and no 100 or 500 ms duration signal trials). The dynamic stimulus range (i.e., fact that stimuli of variable length - 25, 200, 500 ms - are presented pseudo-randomly) in the basic task increases attentional demand by introducing uncertainty of target presentation. The ability of rats to detect signals has been shown to decrease as signal duration decreases (McGaughy and Sarter, 1995). A session comprised entirely of short duration signals could therefore represent an increased attentional demand, due to decreased salience of the signal (Muir et al., 1994). However, in a session that contains only 25 ms signals, rats demonstrate improved performance to detect signals (as well as a small improvement

in performance to reject signals) than when the 25 ms signals are presented embedded in a dynamic stimulus range, and this is thought to be because the decrease in uncertainty (of signal duration) leads to a decrease in attentional demand (Newman and McGaughy, 2008).

Predictable tone

A pulse tone (cross-modal) distractor was used at a frequency of 0.5 hertz. The ability of rats to detect signals has been shown to be improved by the presence of a consistent (predictable) frequency tone of 0.5 hertz, when attentional demands are high. It is thought that this is due to the rats using the tone to time when to attend (Newman and McGaughy, 2008). It was therefore expected that the predictable auditory tone session would represent decreased attentional demand when compared to the basic task. It was therefore expected that the predictable auditory distractor would facilitate the signal detection for control rats.

Predictable light distractor

The house light was flashed at a frequency of 0.5 hertz to provide a uni-modal distractor. Unlike in the presence of the predictable auditory (cross modal) distractor, the ability of rats to detect and correctly reject signal and non-signal trials has been shown to be impaired by the presence of a consistent (predictable) frequency light of 0.5 hertz (Newman and McGaughy, 2008). It was therefore expected that the predictable light distractor session would represent increased attentional demand when compared to the basic task.

Irregular light distractor

The house light was flashed at a varying duration and rate of presentation throughout the session (either 500, 100 or 25 ms on-off) to provide an unpredictable uni-modal distractor. This type of visual distractor has been shown to impair the ability of rats to detect and correctly reject signal and non-signal trials, in a similar way to the predictable light distractor (McGaughy et al., 1996). Because of the combination of the potent distractor and the decrease in predictability (the variation in duration and rate of presentation), it was expected that the irregular light distractor session would represent the greatest challenge to attentional performance.

Behavioural measures recorded during performance of all phases of the SAT included signal detection (a percent correct measure: number of hits / number of hits plus number of misses multiplied by 100), correct rejections (a percent correct measure: number of correct rejections / number of correct rejections plus number of false alarms multiplied by 100) and the number of omissions for signal and non-signal trials separately.

Experiment 2: Attentional set shifting

Apparatus

Attentional set shifting performance was tested using terra-cotta pots that rats were taught to dig in to receive a food reward. The pots were 10cm high with an internal diameter of 10.2cm. The insides of the pots were filled with digging media which were scented using diluted aromatherapy oils (essential oils diluted 1:100 in vegetable oil).

The outsides of the pots were covered with textures. Each pot therefore contained up to three stimulus types (digging media, odour and texture). Pot(s) were placed into one end of a rectangular plastic testing box (91.4cm long, 45.7cm wide and 25.4cm high). A removable divider separated the end of the box that contained the pot(s) and a holding area (30cm long) where the rat was held prior to initiation of the trial. The purpose of the removable divider was to control precisely the onset of the task, without the potential bias of placing the rat into the box after the pot(s) had been placed.

Food reward for all stages of training and testing was a quarter piece of Cheerios breakfast cereal, and the unabated pot always contained an equal but crushed amount of food reward to act as an odour mask, and prevent the rat from using the scent of the cereal to identify the correct stimulus.

Shaping (day 1)

The rat was placed in the holding area of the testing box whilst an unscented pot filled with rodent bedding material, with a food reward placed on top of the bedding, was placed at the other end of the box. The divider was removed and a timer begun. The rat was then allowed 90s to retrieve the food reward. Once the food reward was collected or at 90s, the pot was removed from the box and the rat returned to the holding area. The procedure was then repeated and the position of the pot was pseudorandomly varied from the right and left side of the box to prevent development of a response side bias. Once the rat had retrieved 10 unburied food rewards (consecutively with forelimb dig), the food reward was buried at increasing depths until the rat completed ten consecutive trials using the forelimb to dig for a fully buried reward.

Exemplar training (day 2)

Rats were given a series of discriminations to perform, in order to introduce the types of stimuli that would be tested in the IDED task (digging media, odour and texture). For this stage, 2 pots were placed in the box. Both pots contained one stimulus type e.g., odour. Two different exemplars of the stimulus (e.g., for odour, one pot would be scented with pine and the other with black cherry) were used and the rat was consistently rewarded for digging in the same one of the two exemplars. Each rat was required to reach a criterion of six consecutive correct choices for each of the three stimulus types (dimensions) (see Table 6.2 for pairs of stimuli used).

Exemplars were as follows, digging media: shredded green tissue paper vs shredded white tissue paper; odour: pine vs black cherry; texture: white Vetbed (faux sheep skin) vs the reverse side of the Vetbed. For odour and texture discriminations, both pots were filled with shredded cardboard to hide the reinforcer.

Stage	**Odour pairs**	**Digging media pairs**	**Texture pairs**
Exemplar training (day 2)	Pine vs black cherry	Shredded green tissue paper vs shredded white tissue paper	Vetbed (faux sheep skin) vs reverse side Vetbed
IDED Discriminations (day 3)	Cinnamon vs patchouli Rose vs vanilla Jasmine vs gardinia	Light foam shapes vs dark foam shapes Plastic beads vs gravel Buttons vs Shavings	Felt vs reverse side aluminum foil Smooth side corduroy vs ribbed side corduroy Flanel vs bubble wrap

Table 6.2 Pairs of stimuli used in Experiment 2. Stimulus pair combinations and sequences were counterbalanced as far as possible for prior anaesthetic exposure and sex, such that no two rats of the same sex within a group received the same combinations in the same order.

Intra-dimensional / extra-dimensional attentional set shifting task (day 3)

IDED testing consisted of the following phases in the following order: the simple discrimination (SD), compound discrimination (CD), compound reversal (CDR), intra-dimensional shift (ID), intra-dimensional shift reversal (IDS), extra-dimensional shift (ED) and extra-dimensional shift reversal (EDS). Pairs of stimuli used on day 3 differed from those used during exemplar training (see Table 6.2). For each of these phases the rat was allowed four discovery trials, where the rat had 90 s to explore both pots. If the rat made an incorrect choice (dug in the wrong pot), an incorrect choice was recorded but the rat was allowed to search in the correct pot and retrieve the food reward. Rats were allowed to dig until the food reward was collected following all correct choices. Choice latency (time between removal of the divider and first digging behaviour - in either the correct or incorrect pot) was recorded and during non- discovery trials rats were allowed up to 60 s to make a response. If either an incorrect response was made or 60 s expired (in which case an omission was recorded) the trial was aborted and the rat returned to the holding area. Trials continued for each phase until the criterion level of six consecutive correct responses was made. The number of trials taken to reach criterion, for each phase, was recorded.

Simple discrimination

The simple discriminations tested between 2 pots that differed on only one dimension (stimulus type - digging media, odour or texture). For example, in a test of odour discrimination a pot scented with cinnamon was baited with a food reward and presented in combination with a pot scented with patchouli. Digging media and texture were identical for both pots. Alternate pairs of pots were tested where one dimension, e.g., digging media, varied between pairs. For example, cinnamon / light

foam shapes vs patchouli light foam shapes would alternate with cinnamon / dark foam shapes vs patchouli / dark foam shapes, and the pot containing cinnamon was rewarded in each case, regardless of the digging media type. In this way rats would learn that the digging media were irrelevant.

Compound discrimination

In the compound discriminations the rewarded stimulus remained constant (as in the simple discriminations), but the pots now differed on two dimensions rather than one. Alternate testing pairs would be, e.g., cinnamon / light foam shapes vs patchouli / dark foam shapes and cinnamon / dark foam shapes vs patchouli / light foam shapes, where the pot containing cinnamon was rewarded in each case, regardless of the digging media type. In this example, rats would therefore have to ignore digging media (as in the simple discriminations) but would be presented with 2 pots that differed in two dimensions (odour and digging media) not just the relevant one (odour).

Compound discrimination reversal

For the reversal trials the rat was reinforced for responding to the previously unrewarded exemplar of a dimension, e.g., patchouli odour. Otherwise, the testing pairs were identical to those used in the compound discriminations.

Intra-dimensional shift

A complete new set of stimuli was introduced in the ID phase. However, the same dimension that predicted reward in the SD, CD and CDR (e.g., odour) predicted reward in the ID. Rats were therefore required to shift attention to a novel stimulus

within the same dimension as previous phases, i.e., make an intra-dimensional shift in attention.

Intra-dimensional shift reversal

For the IDS reversal trials the rat was reinforced for responding to the previously unrewarded exemplar of a dimension. Otherwise, the testing pairs were identical to those used in the intra-dimensional discriminations.

Extra-dimensional shift

In the extra-dimensional shift the previously irrelevant dimension, e.g., digging media, now predicted reward. Rats were therefore required to shift attention to a stimulus within a different dimension, i.e., make an extra-dimensional shift in attention.

Extra-dimensional shift reversal

For the EDS reversal trials the rat was reinforced for responding to the previously unrewarded exemplar of a dimension. Otherwise, the testing pairs were identical to those used in the extra-dimensional discriminations.

There were six possible sequences of shifts between dimensions in the ID and ED phases (digging media to texture, odour to digging media, texture to digging media, odour to texture, digging media to odour, texture to odour). The first four of these possible sequences were used and rats were pseudorandomly assigned a sequence of testing, balanced as far as possible for prior anaesthesia exposure and sex. For an

example of a possible combination of pairs of stimuli for a rat shifting from odour to digging media see Table 6.3.

Behavioural measures recorded during each of the phases were: the number of trials taken to reach criterion of six consecutive correct choices (trials to criterion); the time taken to make a correct choice (correct choice latency) and the time taken to make an incorrect choice (incorrect choice latency).

	Testing pair 1	**Testing pair 2**	**Irrelevant stimulus type**
SD	Cinnamon* / light foam shapes vs patchouli light foam shapes	Cinnamon* / dark foam shapes vs patchouli dark foam shapes	Felt
CD	Cinnamon* / light foam shapes vs patchouli dark foam shapes	Cinnamon* / dark foam shapes vs patchouli / light foam shapes	Felt
CDR	Patchouli* / light foam shapes vs cinnamon / dark foam shapes	Patchouli* / dark foam shapes vs cinnamon / light foam shapes	Felt
ID	Rose* / plastic beads vs vanilla / gravel	Rose* / gravel vs vanilla / plastic beads	Reverse side aluminium
IDR	Vanilla* / plastic beads vs rose / gravel	Vanilla* / gravel vs rose / plastic beads	Reverse side aluminium
ED	Buttons* / jasmine vs shavings / gardinia	Buttons* / gardinia vs shavings / jasmine	Ribbed side corduroy
EDR	Shavings* / jasmine vs buttons / gardinia	Shavings* / gardinia vs buttons / gardinia	Ribbed side corduroy

*Table 6.3 Example of a possible combination of pairs of stimuli for a rat shifting from odour to digging media. Note that odour to digging media is one of four possible sequences used. SD, simple discrimination; CD, compound discrimination; CDR, compound discrimination reversal; ID, intradimensional shift; IDR, intradimensional shift reversal; ED extradimentional shift; EDR, extradimensional shift reversal. *The rewarded/correct choice.*

Statistical analysis

Anaesthesia

Blood gas data from groups P7, P13 and C were compared using one-way analysis of variance (ANOVA) with group (3 levels) as the between subjects factor. Fisher LSD post hoc analysis was carried out.

Experiment 1: Sustained attention task

Baseline data from each of the five baseline sessions (one session before testing on each task variant) were averaged and group performance compared using repeated measures ANOVA, with block (3 levels: first, middle and last block of 54 trials) and signal length (signal trial parameters only) (3 levels: 500, 100 and 25 ms) as the within-subjects factors, and anaesthesia group (3 levels) plus sex (2 levels) as the between-subjects factors. Tukey's post hoc analysis was performed.

Data for each of the task manipulations were compared using repeated measures ANOVA, with phase (2 levels: 1 session of baseline data and 1 session of data on manipulated task), block (3 levels: first, middle and last block of 54 trials) and signal length (signal trial parameters only and *not* for 'Short duration stimulus' task variation) (3 levels: 500, 100 and 25 ms) as the within-subjects factors, and anaesthesia group (3 levels) plus sex (2 levels) as the between-subjects factors. Tukey's post hoc analysis was performed in all cases.

Experiment 2: Attentional set shifting

Data for trials to criterion and correct choice latency were compared using repeated measures ANOVA, with phase (7 levels: SC, CD, CDR, ID, IDR, ED and EDR) as the within-subjects factor and group (3 levels: control condition [group C], single exposure to anaesthesia [group 1A] and repeated exposures to anaesthesia [group 3A]) and sex (2 levels) as between-subjects factors. Tukey's post hoc analysis was performed.

Some rats made zero incorrect choices and so data for incorrect choice latency were only analysed for the extra-dimensional shift (because only one rat made zero incorrect choices for this phase). A one-way ANOVA with multiple factors (a univariate general linear model) was constructed with EDS as the dependent variable and group (3 levels: control condition [group C], single exposure to anaesthesia [group 1A] and repeated exposures to anaesthesia [group 3A]) and sex (2 levels) as the independent variables.

The sequence that rats were required to discriminate stimuli (e.g., odour in the ID to digging media in the ED) was discounted as a variable in the analysis of attentional set shifting data. The number of trials taken to reach criterion during exemplar training was analysed using repeated measures ANOVA with dimension (3 levels: odour, digging media and texture) as the within-subjects factor and group (3 levels) and sex (2 levels) as the between-subjects factors. The ability of rats to discriminate stimuli did not depend on the dimension of the stimuli, demonstrating that the three dimensions are similar in difficulty to discriminate (there was no main effect of dimension ($F[2,38] = 1.192$, $p = 0.315$) and no interaction of dimension with other

factors (all F values < 1.7, all p values > 0.17)). In addition, the effect of the sequence of shift between dimensions that each rat was required to follow (e.g., odour to digging media) was analysed to ensure that there was no inherent difference in the difficulty of the four sequences used. A repeated measures ANOVA was constructed for trials to criterion data from the IDED testing phases with phase (7 levels: SC, CD, CDR, ID, IDR, ED and EDR) as the within-subjects factor and sequence (4 levels) as the between-subjects factor. The ability of rats to discriminate stimuli did not depend on the sequence of shift between dimensions, as demonstrated by the lack of a main effect of sequence ($F[3,21] = 0.392$, $p = 0.76$) and lack of an interaction of sequence and phase ($F[3,21] = 0.605$, $p = 0.615$). The variable 'sequence' was therefore omitted from analysis for the attentional set shifting task.

Results

Anaesthetic physiology

Body temperature was successfully maintained between 36C and 37C, and blood oxygenation was successfully maintained above 95%, in all anaesthetised pups (those anaesthetised before behavioural testing and those anaesthetised before blood gas analysis). Both anaesthesia groups were mildly hypercapnic compared to controls. There were no other group differences in blood gas parameters. The supporting statistical analyses for these statements can be found below.

Blood gas data from the three groups were compared using one-way ANOVA with anaesthetic group (3 levels) as the between-subjects factor. Table 6.4 shows the average group values for blood pH, pCO_2 and pO_2. Both anaesthetised groups demonstrated mild relative acidaemia (decrease in blood pH) compared to the control group; whilst this effect is expected, it did not reach statistical significance ($F[2,11] = 3.56$, $p = 0.072$), suggesting that the study may have been underpowered. There was a main effect of group for pCO_2 ($F[2,11] = 5.39$, $p = 0.029$) which reflected that both anaesthetic groups were mildly hypercapnic (increased partial pressure of carbon dioxide within the blood) compared to control rats (pairwise comparisons of group C vs group 1A: $p = 0.012$; group C vs group 3A: $p = 0.038$). There was no group difference in pO_2 ($F[2,11] = 0.031$, $p = 0.970$).

Parameter	group C	group 1A	group 3A
pH	7.41 +/- 0.03	7.30 +/- 0.02	7.34 +/- 0.02
pCO2 (mmHg)	36 +/- 2	54 +/- 4	50 +/- 5
pO2 (mmHg)	101 +/- 7	103 +/- 19	99 +/- 7

Table 6.4 Average physiological parameters for control rats (group C: brief immobilisation with 6% sevoflurane and 30% oxygen), rats anaesthetised with 2.8% sevoflurane and 30% oxygen at P7 group 1A) or rats anaesthetised with 2.8% sevoflurane and 30% oxygen at P7, P10 and P13. Values are presented as mean +/- sem.

Experiment 1: effect of anaesthetic exposure during development on performance of the SAT during adulthood

The results of the SAT manipulations do not provide compelling evidence of an effect of developmental exposure to sevoflurane (either single or repeated) on attention. Effects of prior anaesthetic exposure were detected during the short duration session and the unpredictable light distractor session. However, in both cases these effects were higher order interactions that are difficult to interpret. A full description of the results is given below.

Baseline behavioural testing performance

Prior to performance of each task manipulation rats were required to reach criterion (>75% hits to 500ms signals and >75% correct rejections to non-signal trials) on the basic task for two consecutive days. Performance during the second of these two days at criterion was taken as baseline performance for that particular task manipulation, i.e., five baseline performance days in total, each of which directly preceded a testing day (on a task variation that was designed to alter the attentional demands of the task). The average values of the five baseline performance days were calculated and analysed. Given that rats were trained to criterion, group differences in performance were not expected in these analyses.

Behavioural measures recorded included the number of days taken to initially reach criterion on the basic task, the percentage of signals detected, the percentage of signals correctly rejected and the number of omitted trials.

Due to a technical difficulty, the number of days taken to reach criterion on the basic task was only recorded for 24 out of 30 rats. Rats took a similar number of days to reach criterion on the basic task, regardless of whether they were female or male (one-way ANOVA with 'number of days to criterion' as the dependent variable and sex as the between-subjects factor: $F[1,23] = 0.093$, $p = 0.763$) (mean +/- sem number of days to criterion females: 25.5 +/- 2.7, males: 23.67 +/- 5.35), and regardless of prior anaesthetic exposure (one-way ANOVA with 'number of days to criterion' as the dependent variable and anaesthetic group as the between-subjects factor: $F[2,23] = 2.01$, $p = 0.16$) (mean +/- sem number of days to criterion group C: 19.63 +/- 1.68, group 1A: 22.5 +/- 7.8, group 3A: 21.63 +/- 2.83).

The baseline performance of rats did not differ depending on group (i.e., did not differ depending whether rats had been exposed to anaesthesia during development or not). As expected, the ability of rats to detect signals decreased with decreasing signal duration, i.e., rats detected a lower percentage of signals when a more brief signal was presented, and as the sessions progressed, i.e., as rats potentially became both satiated and fatigued. The supporting statistical analyses for these statements can be found below.

Baseline signal detection

Data for the percentage of signal trials that were correctly detected were analysed using repeated measures ANOVA with block (3 levels: first, middle and last block of

54 trials) and signal duration (3 levels: 500, 100 and 25 ms) as within-subjects factors and anaesthesia group (3 levels: group C [n=10], group 1A [n=10] group 3A [n=10]) and sex (2 levels) as the between-subjects factors.

As expected, the ability of all rats to detect signals decreased with increasing time on task (main effect of block: $F[2,48] = 4.55$, $p = 0.016$; block 1: 62.2 +/- 0.15, block 2: 60.2 +/- 0.16 and block 3: 57.9 +/- 0.18, pairwise comparison block 1 vs block 3 $p = 0.022$). This vigilance decrement is a key feature of SAT performance (Sarter and McGaughy, 1998). The performance of all rats also decreased as signal duration decreased (main effect of signal duration: $F[2,48] = 458.3$, $p < 0.005$; mean percentage of correct signal detections +/- sem at 500, 100 and 25 ms: 82.4 +/- 0.11, 56.8 +/- 0.19 and 41.1 +/- 0.18 respectively; all pairwise comparisons $p < 0.005$).

Rats detected a similar percentage of signals regardless of whether they were female or male and regardless of prior anaesthesia exposure (main effect of group: $F[2,24] = 0.237$, $p = 0.791$; main effect of sex: $F[1,24] = 2.47$, $p = 0.129$) (mean +/- sem percentage signal detection group C: 59.1 +/- 2.5, group 1A: 59.9 +/- 2.5, group 3A: 61.4 +/- 2.5). There were no interactions of anaesthesia group with other factors, for signal detection, and no higher order interactions (all F values < 2.48; all p values > 0.109).

Baseline correct rejections

Data for the percentage of correctly rejected non-signal trials were analysed using repeated measures ANOVA with block (3 levels: first, middle and last block of 54 trials) as the within-subjects factor and anaesthesia group (3 levels: group C [n=10], group 1A [n=10], group 3A [n=10]) and sex (2 levels) as the between-subjects factors.

Rats rejected a similar percentage of non-signal trials regardless of whether they were female or male and regardless of prior anaesthesia exposure (main effect of group: $F[2,24] = 0.077$, $p = 0.926$; main effect of sex: $F[2,24] = 0.049$, $p = 0.827$). There were no interactions of group with other factors, for correct rejections, and no higher order interactions (all F values < 1.39; all p values > 0.43). All rats demonstrated a small but statistically significant increase in the percentage in correctly rejected non-signal trials during the middle block of trials (main effect of block: $F[2,48] = 7.685$, $p = 0.001$) (block 1: 79.2 +/- 0.08, block 2: 82.2 +/- 0.08 and block 3: 78.3 +/- 0.12; pairwise comparisons of block 2 with block 1 and block 3: $p = 0.002$ and $p = 0.002$). It is not clear why the performance of rats improved during the middle block of trials, as there was no difference in demands of the task during this block and it would usually be expected that performance would decrease as the session progresses.

Baseline omissions

Exposure to anaesthesia did not cause rats to omit more signal or non-signal trials than control rats during baseline testing. Many rats (from each of the three groups) made zero omissions during sessions and therefore the number of omitted trials was averaged across signal duration (for signal trial omissions) and testing block, resulting in a single figure per rat for signal trial omissions, and a single figure per rat for non-signal trial omissions. Data were found to be non-normally distributed. Homogeneity of variance was tested using one way ANOVA with absolute difference (the difference between the rank and the mean of rank for each data point) as the dependent and anaesthetic group as the factor. Group variances were found to be equal for both signal omissions and non-signal omissions (both F values < 3.1; both p values > 0.061); although there was a trend towards unequal group variance for non-signal trials, the

data demonstrated similarly shaped distributions and therefore a Kruskal-Wallis test was used to analyse baseline signal and non-signal omissions separately. There was no group difference in signal trial omissions ($H[2] = 0.516$, $p = 0.773$) or non-signal trial omissions ($H[2] = 0.082$, $p = 0.96$).

Baseline data used for comparison with testing session data

Please note that average baseline performance values (as analysed above) were not used as the baseline performance for comparison with the five task manipulation sessions, but rather the baseline session that immediately preceded the particular testing session was used.

Task manipulation testing performance: High event rate session

For this task variation, the inter-trial interval was 9 seconds +/- 3 seconds for all trials, rather than being 12 seconds +/- 3 seconds. Baseline performance consisted of one basic task session (intertrial interval 12 seconds +/- 3s), 24 hrs prior to testing. Testing performance consisted of one session of 162 trials.

Exposure to anaesthesia during development did not impair the ability of rats to detect or reject more rapidly presented signals, whilst performing the high event rate session. In other words, there was no effect of anaesthesia on signal detection (mean +/- sem percentage signal detection group C: 60.4 +/- 2.6, group 1A: 57.8 +/- 2.6, group 3A: 58.2 +/- 2.6) or correct rejections (mean +/- sem percentage correct rejection group C: 81 +/- 2.5, group 1A: 82.3 +/- 2.5, group 3A: 82.3 +/- 2.5) during the high event rate session. Exposure to anaesthesia during development did not cause rats to make

more omissions during the high event rate session. The supporting statistical analysis for these statements can be found below.

High event rate signal detection

Repeated measure ANOVA was used to analyse signal detection data with phase (2 levels: baseline testing and high event rate session), signal duration (3 levels: 500, 100 and 25 ms) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

Exposure to anaesthesia did not impair the ability of rats to perform during the high event rate session. There was no main effect of group or sex for signal detection during the high event rate task manipulation (group: $F[2,24] = 0.299$, $p = 0.744$) and no interactions of group with any factor (all F values < 1.39; all p values > 0.15). As expected, the ability of rats to detect signals decreased as signal duration decreased (main effect of signal duration: $F[2,48] = 170.825$, $p < 0.005$; mean +/- sem 500 ms: 80.7 +/- 1.7, 100 ms: 55.5 +/- 2.3, 25 ms: 40.2 +/- 1.8; all pairwise comparisons $p < 0.005$). Unexpectedly, there was a small increase in the percentage of signals detected during the testing phase session compared to the baseline session (main effect of phase: $F[1,24] = 3.44$, $p = 0.076$) (mean +/- sem baseline: 57.2 +/- 1.9, testing session: 60.4 +/- 1.5) and there was an interaction between phase and signal duration (phase by signal duration interaction: $F[2,48] = 8.42$, $p = 0.001$). With rats detecting a higher percentage of 500ms signals and a lower percentage of 25ms signals during the baseline phase compared to the high event rate phase (Fig 6.1).

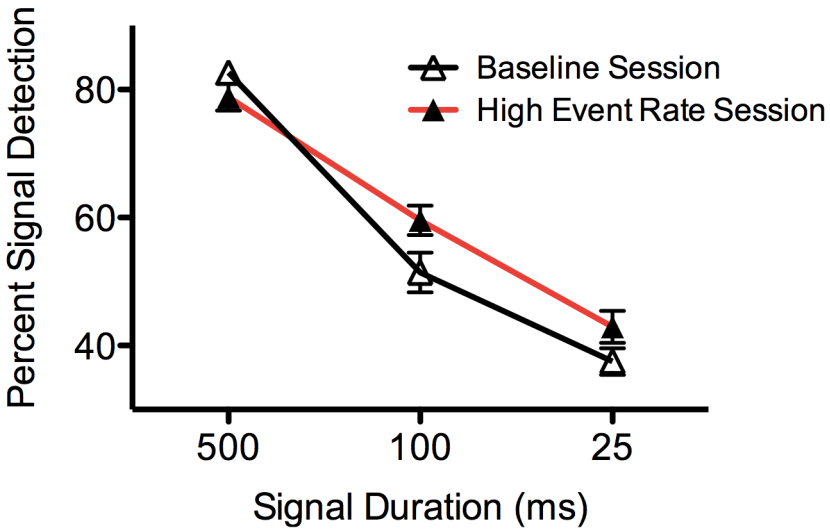

Figure 6.1 Percentage of signals detected during the baseline (black, open triangles) and the high event rate session (red, closed triangles), for each of the three signal durations within a session. Rats detected a higher percentage of 500 ms signals, and a lower percentage of 25 ms signals during the baseline session, compared to the high event rate session. Data are represented as mean +/- sem.

High event rate correct rejections

Repeated measure ANOVA was used to analyse correct rejection data with phase (2 levels: baseline testing session and high event rate session) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

Exposure to anaesthesia did not impair the ability of rats to correctly reject non-signal high event rate trials. There were no main effects of group or sex for correct rejections for the high event rate task manipulation (group: $F[2,24] = 0.076$, $p = 0.927$; sex: $F[1,24] = 0.829$, $p = 0.371$) and no interactions of group with any other factor (all F values < 2.1; all p values > 0.097). There were no other effects or interactions for correct rejection performance (all F values < 2.75; all p values > 0.074).

Task manipulation testing performance: Short signal duration session

For this task variation, signal trials consisted entirely of 25 ms duration signals, rather than a dynamic stimulus range of 500, 100 or 25 ms duration signals. Baseline performance consisted of one basic task session (with variable signal duration) 24 hrs prior to testing; however, only data for the 25 ms signal trials were compared with testing phase performance. Testing phase performance consisted of one session of 162 trials (81 signal trials and 81 non-signal trials).

Exposure to anaesthesia during development did not alter the ability of rats to detect short duration signals when they were not embedded within a dynamic stimulus range (mean +/- sem percentage signal detection group C: 45.3 +/- 4.4, group 1A: 44.5 +/- 4.4, group 3A: 48.8 +/- 4.4). However, the effect of anaesthesia on correct rejections differed depending on whether rats were performing the basic or manipulated task, and on the amount of time spent on task (block). Exposure to anaesthesia during development did not alter the number of omissions that rats made during the short signal duration session. The supporting statistical analyses for these statements can be found below.

Short signal duration signal detection

Repeated measure ANOVA was used to analyse signal detection with test phase (2 levels: baseline testing session and short signal duration only session) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

Exposure to anaesthesia did not alter the ability of rats to correctly detect short signal duration signals. There were no main effects of group or sex for signal detection for the short signal duration task manipulation (group: $F[2,24] = 0.264$, $p = 0.77$; sex: $F[1,24] = 0.192$, $p = 0.665$) and no group by sex interaction ($F[2,24] = 0.455$, $p = 0.640$). There were no interactions of group with any other factor (all F values < 1.81; all p values > 0.175). As would be predicted by the decrease in unpredictability (Yu and Dayan, 2005) of the 25ms signal session, all rats detected a higher percentage of 25 ms signals during the test phase than during the baseline phase (main effect of phase: $F[1,24] = 22.077$, $p < 0.005$; mean +/- sem baseline: 40.4 +/- 2.6, short signal duration session: 52 +/- 3) (Fig 6.2). There were no other effects or interactions (all F values < 1.47; all p values > 0.24).

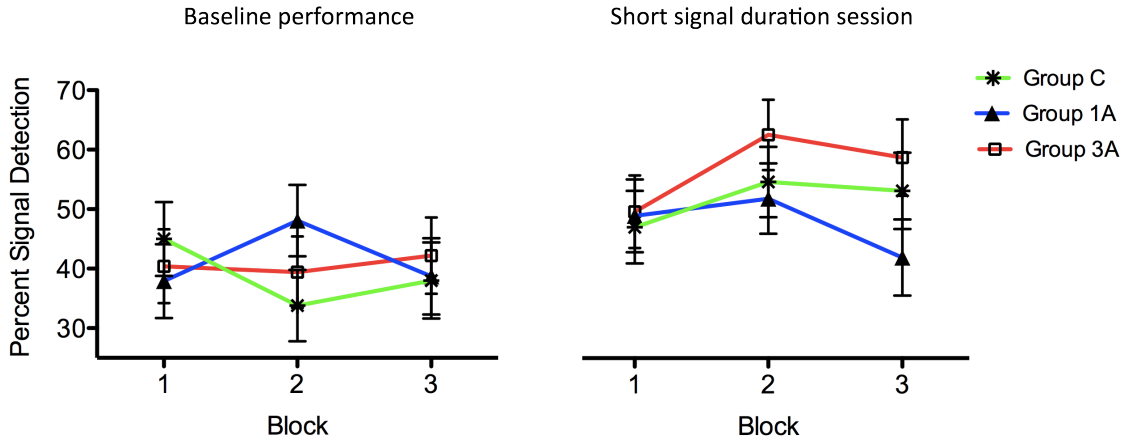

Figure 6.2 Percentage of signals detected during the baseline (left) and the short signal duration only session (right), for each of the three blocks of trials within a session. Rats detected a higher percentage of signals during the short signal duration session than during the baseline session. Data are represented as mean +/- sem.

Short signal duration correct rejections

Repeated measures ANOVA was used to analyse correct rejections with phase (2 levels: baseline testing session and short signal duration only session) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

The ability of rats to correctly reject non-signal trials depended on prior anaesthesia exposure, as well as whether they were performing the basic or manipulated task, and the amount of time spent on task (block of the session). In other words, there was a phase by block by group interaction ($F[4,48] = 4.014$, $p = 0.007$) (see Fig 6.3). This interaction occurred despite the absence of main effects of group or sex for correct rejections for the short signal duration task manipulation (group: $F[2,24] = 0.071$, $p = 0.932$; sex $F[1,24] = 1.57$, $p = 0.22$) or a group by sex interaction ($F[2,24] = 0.281$, $p = 0.758$). The interaction of anaesthesia group with phase and block was decomposed initially by examining the significance of the phase by group

interactions for each of the three blocks separately. The phase by group interaction was significant only for block 2 ($F[1,27] = 4.161$, $p = 0.027$). Secondarily the significance of the phase by block interaction for each group separately was examined and was found to be significant for group C ($F[2,18] = 7.823$, $p = 0.004$). The reason for the interaction between phase, block and group for correct rejection of non-signal trials is unclear, however taken together with examination of Figure 6.2, it would seem that the interaction is driven by the decrease in performance of group C, during block 2 of the short duration signal session. Given that all rats detected a higher percentage of signals during this session (see Fig 6.2 above), it could be that group C are off setting this improvement in signal detection against a deterioration in signal rejection (see Fig 6.3 below), whereas the anaesthesia groups are not.

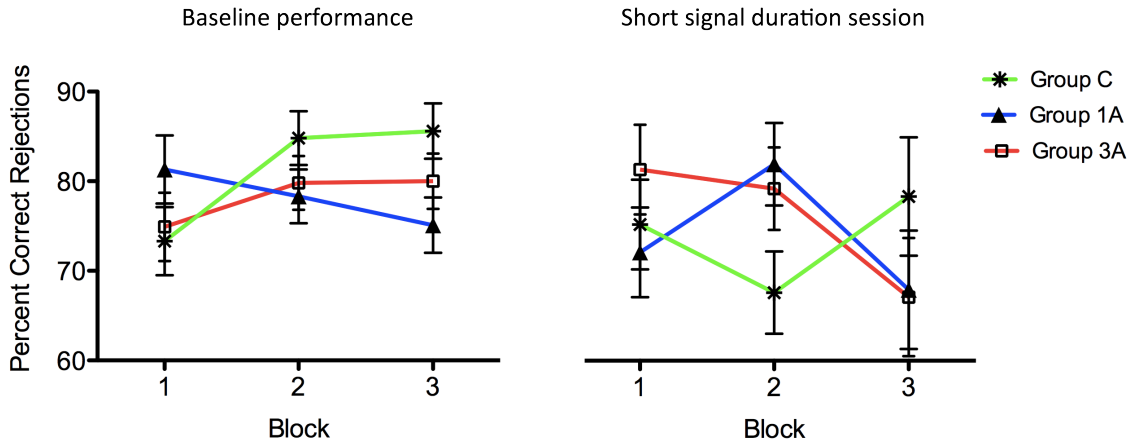

Figure 6.3 Percentage of correct rejections made during the baseline task session (left) and the short signal duration only session (right), for each of the three blocks of trials within a session. The ability of rats to correctly reject non- signal trials depended on prior anaesthesia exposure, as well as whether they were performing the basic or manipulated task and the stage (block) of the task that they were performing (there was a group by phase by block interaction). Data are represented as mean +/- sem.

Task manipulation testing performance: Predictable tone session

For this task variation, a pulse tone distractor was used at a frequency of 0.5 Hz. Baseline performance consisted of one basic task session 24 hrs prior to testing. Testing performance consisted of one session of 162 trials.

Exposure to anaesthesia during development did not alter the ability of rats to detect signals (mean +/- sem percentage signal detection group C: 53 +/- 3.2, group 1A: 56.4 +/- 3.2, group 3A: 56 +/- 3.2), or to correctly reject non-signal trials (mean +/- sem correct rejections group C: 78.3 +/- 2.1, group 1A: 74.1 +/- 2.1, group 3A: 79.2 +/- 2.1), in the presence of a predictable tone distractor. The supporting statistical analysis for these statements can be found below.

Predictable tone signal detection

Repeated measures ANOVA was used to analyse signal detection data, with phase (2 levels: baseline testing and predictable tone distractor session), signal duration (3 levels: 500, 100 and 25 ms) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

There was no effect of anaesthesia on the ability of rats to detect signals in the presence of a predictable tone distractor. There were no main effects of group or sex for signal detection for the predictable tone distractor task manipulation (group: $F[2,24] = 0.34$, $p = 0.967$; sex: $F[1,24] = 0.928$, $p = 0.345$) and no interactions of group with any other factor (all F values < 1.39, all p values > 0.253). Rats detected a similar

percentage of signals in the presence of the predictable tone distractor as they did in the absence of it (main effect of phase: $F[1,24] = 0.006$, $p = 0.938$). As expected, rats detected a lower percentage of signals when signal duration was shorter (main effect of signal duration: $F[2,48] = 222.09$, $p < 0.005$) (mean +/- sem 500 ms: 80.6 +/- 1.6, 100 ms: 57.7 +/- 2.2, 25 ms: 41.5 +/- 1.9; all pairwise comparisons $p < 0.005$) but this did not differ depending on the presence of the predictable tone distractor (phase by signal duration interaction $F[2,48] = 0.347$, $p = 0.709$). The performance of rats did not decline as the sessions progressed (main effect of block: $F[2,48] = 1.927$, $p = 0.157$) and there was no interaction of phase and block ($F[2,48] = 1.069$, $p = 0.352$).

Predictable tone correct rejections

Repeated measure ANOVA was used to analyse correct rejection data, with phase (2 levels: baseline testing and predictable tone distractor session) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

There was no effect of anaesthesia on the ability of rats to correctly reject signals in the presence of a predictable tone distractor. There were no main effects of group or sex for correct rejections for the predictable tone distractor task manipulation (group: $F[2,24] = 1.75$, $p = 0.195$; sex: $F[1,24] = 0.375$, $p = 0.546$) and no interaction of group with sex ($F[1,24] = 1.06$, $p = 0.363$). Rats rejected a similar percentage of non-signal trials in the presence of the predictable tone distractor as they did in the absence of it (main effect of phase: $F[1,24] = 0.805$, $p = 0.378$). There was a main effect of block ($F[2,48] = 4.054$, $p = 0.024$), that reflected superior performance during the middle

block of trials (block1: 75.6 +/- 2, block 2: 80.6 +/- 1.2, block 3: 75.4 +/- 1.6; pairwise comparison of block 2 vs block 1 and block 3: $p = 0.005$ and $p = 0.003$ respectively). It is not clear why rats would perform better during the middle block of trials but this effect was seen in baseline performance also.

Task manipulation testing performance: Predictable light distractor session

For this task variation, the house light was flashed at a frequency of 0.5 Hz. Baseline performance consisted of one basic task session 24 hrs prior to testing. Testing performance consisted of one session of 162 trials.

Exposure to anaesthesia during development did not impair the ability of rats to detect signals, or correctly reject non-signal trials, in the presence of a predictable light distractor. In other words, there was no effect of anaesthesia on signal detection (mean +/- sem group C: 59 +/- 2.9, group 1A: 57.7 +/- 2.9, group 3A: 59.1 +/- 2.9) or correct rejections (mean +/- sem group C: 70.9 +/- 2.7, group 1A: 75.9 +/- 2.7 and group 3A: 77 +/- 2.7) during the predictable light distractor session. Exposure to anaesthesia during development did not alter the number of omissions made by rats during the predictable light distractor session. The supporting statistical analysis for these statements can be found below.

Predictable light distractor signal detection

Repeated measures ANOVA was used to analyse signal detection data, with phase (2 levels: baseline testing and short signal duration only session), signal duration (3 levels: 500, 100 and 25 ms) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to

sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

Exposure to anaesthesia did not impair the ability of rats to detect signals in the presence of a predictable light distractor. There was no main effect of group or interaction of group with other factors for signal detection for the predictable light distractor task manipulation (main effect of group: $F[2,24] = 0.07$, $p = 0.931$); all group interaction F values < 1.72, all p values > 0.19). As expected, rats detected fewer signals during the testing session compared to the baseline session (main effect of phase: $F[1,24] = 16.17$, $p < 0.005$; mean +/- sem baseline: 63.2 +/- 1.6, testing session: 54 +/- 2.3), as the sessions progressed (main effect of block: $F[2,48] = 4.327$, $p = 0.019$; mean +/- sem block 1: 62.6 +/- 2, block 2: 56.3 +/- 1.9 and block 3: 56.9 +/- 2.5) and with decreasing signal duration (main effect of signal duration: $F[2,48] = 235.57$, $p < 0.005$; mean +/- sem 500 ms: 76.7 +/- 1.8, 100 ms: 55.9 +/- 1.6 and 25 ms: 43.2 +/- 2.2).

Female rats detected fewer signals than males in the presence of a predictable frequency light distractor (main effect of sex: $F[1,24] = 4.24$, $p = 0.05$) (mean percentage signal detection +/- sem females: 55.2 +/- 2.3, males 62 +/- 2.3).

There were interactions between testing phase and block (phase by block interaction: $F[2,48] = 4.88$, $p = 0.012$) and between testing phase and signal duration (phase by signal duration interaction: $F[2,48] = 10.19$, $p < 0.005$), but as neither effect interacted with group or sex no further investigation was carried out.

Predictable light distractor correct rejections

Repeated measures ANOVA was used to analyse correct rejection data, with phase (2 levels: baseline testing and short signal duration only session) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

There was no effect of anaesthesia or sex on the ability of rats to correctly reject non-signal trials. There were no main effects of group or sex on correct rejections (group: $F[2,24] = 1.453$, $p = 0.254$; sex: $F[1,24] = 0.129$, $p = 0.723$) and no interaction of group and sex ($F[2,24] = 0.1.59$, $p = 0.225$). As expected, rats made fewer correct rejections during the testing session, as compared to the baseline session (main effect of phase: $F[1,24] = 15.494$, $p = 0.001$; mean +/- sem baseline: 79.6 +/- 1.2, testing session: 69.6 +/- 2.5) but this effect was predominantly driven by female rats (phase by sex interaction: $F[1,24] = 8.09$, $p = 0.009$; mean +/- sem percent correct rejections for female rats baseline: 82.7 +/- 1.7, testing session: 65.4 +/- 3.5; pairwise comparison $p = 0.001$) rather than male rats (mean +/- sem percent correct rejections for male rats baseline:

76.6 +/- 1.6, testing session: 73.8 +/- 3.7; pairwise comparison $p = 0.28$). There was also an interaction between phase and block (phase by block interaction: $F[2,48] = 5.23$, $p = 0.009$) but as this effect didn't interact with group it was not investigated further.

Task manipulation testing performance: Irregular light distractor session

For this task variation, the house light was flashed at a varying duration and rate throughout the session (either 500, 100 or 25 ms on-off). Baseline performance consisted of one basic task session 24 hrs prior to testing. Testing performance consisted of one session of 162 trials.

The ability of rats to detect signals differed during the baseline and the testing sessions depending on prior exposure to anaesthesia, whether the rats were female or male and whether they were detecting 500, 100 or 25 ms duration signals, i.e., there was a four-way interaction (between phase, group, sex and signal duration). Exposure to anaesthesia did not impair the ability of rats to correctly reject non-signal trials (mean +/- sem percentage correct rejection group C: 78 +/- 2.3, group 1A: 73.8 +/- 2.3, group 3A: 77.7 +/- 2.3), and did not cause rats to omit more trials than control rats, during the irregular light distractor session. The supporting statistical analysis for these statements can be found below.

Irregular light distractor signal detection

Repeated measures ANOVA was used to analyse signal detection data, with phase (2 levels: baseline testing and short signal duration only session), signal duration (3 levels: 500, 100 and 25 ms) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

There was a four way interaction for signal detection, between phase, group, sex and signal duration for signal detection ($F[4,48] = 2.866$, $p = 0.033$) (see Fig 6.3 for a representation of the data). This interaction occurred despite the absence of a main effect of group or sex for signal detection for the irregular light distractor task manipulation (main effect of group: $F[2,24] = 0.332$, $p = 0.72$; main effect of sex: $F[1,24] = 1.152$, $p = 0.294$) and a lack of interaction of group with sex ($F[2,24] = 0.072$, $p = 0.931$). As expected, the performance of all rats was better during the baseline session than the testing session (main effect of phase: $F[1,24] = 12.90$, $p = 0.001$), and deteriorated as signal duration decreased and across the sessions (main effect of signal duration: $F[2,48] = 5.37$, $p = 0.008$, see Fig 6.4; main effect of block: $F[2,48] = 197.122$, $p < 0.005$, mean +/- sem block 1: 73.9 +/- 1.9, block 3: 39 +/- 1.9, pairwise comparison $p < 0.005$). There was also an interaction of phase and block ($F[2,48] = 4.16$, $p = 0.022$) but as this effect did not interact with group it was not explored further.

The interaction of phase, anaesthesia group, sex and signal duration was initially decomposed by examining the simple main effects of signal duration for each group separately. However, this only served to demonstrate that each of the groups detected a lower percentage of signals as signal duration decreased (as expected) (main effect of signal duration group C: $F[2,16] = 52.82$, $p < 0.005$; group 1A: $F[2,16] = 98.4$, $p < 0.005$; group 3A: $F[2,16] = 56.29$, $p < 0.005$). There were no other effects for any group (all F values < 5.12, all p values > 0.055). The higher order interaction detected would therefore appear to reflect group differences in the interaction between phase, sex and signal duration. Further interpretation of this result was limited, as these 3-way interactions were not significant for individual groups (group C: $F[2,16] = 2.545$, $p = 0.11$, group 1A: $F[2,16] = 0.656$, $p = 0.532$, group 3A: $F[2,16] = 0.084$, $p = 0.920$),

and this type of higher order interaction did not occur on any other performance measure.

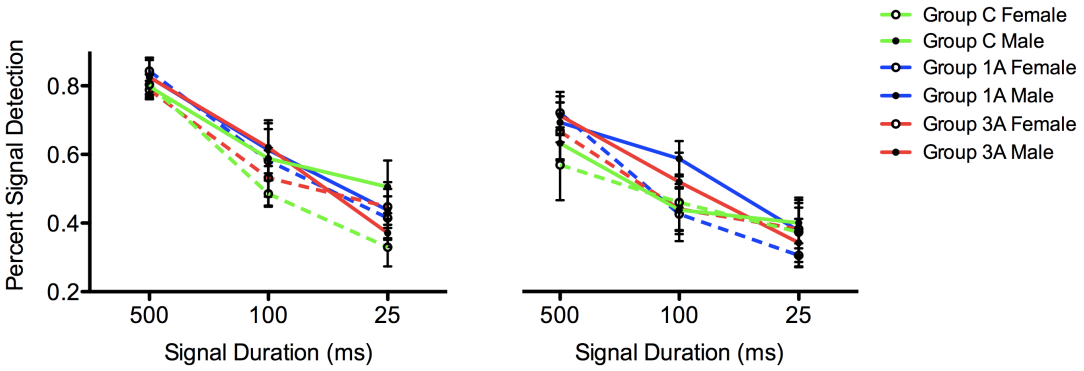

Figure 6.4 Percentage of signals detected during the baseline (left) and the irregular light distractor session (right), for each of the three signal durations. The pattern of signal detection performance differed during the baseline and the testing session, depending on prior exposure to anaesthesia, whether rats were female or male and which duration signal they were detecting (there was a group by phase by sex by signal duration interaction). Data are represented as mean +/- sem.

Irregular light distractor correct rejections

Repeated measure ANOVA was used to analyse correct rejection data, with phase (2 levels: baseline testing and short signal duration only session) and block (3 levels: first, middle and last block of 54 trials) as the within-subjects factors and anaesthesia group (3 levels: control, single exposure to sevoflurane and repeated exposure to sevoflurane) and sex (2 levels) as the between-subjects factors.

Exposure to anaesthesia did not impair the ability of rats to correctly reject non-signal trials in the presence of an unpredictable light distractor. There were no main effects of group or sex for correct rejections for the overlapping light distractor task manipulation (group: $F[2,24] = 0.373$, $p = 0.693$; sex: $F[1,24] = 0.119$, $p = 0.733$) and no interactions of group with any other factor (all F values < 2.16, all p values > 0.089).

As expected, the performance of all rats was better during the baseline session than the testing session (main effect of phase: $F[1,24] = 13.3$, $p = 0.001$). There was an interaction between phase, block and sex (phase by block by sex interaction: $F[2,48] = 3.409$, $p = 0.041$). As these effects did not interact with group they were not explored further.

Omitted trials during the task manipulations

Exposure to anaesthesia did not cause rats to omit more signal or non-signal trials than control rats. Many rats (from each of the three groups) made zero omissions during sessions (regardless of task manipulation) and therefore the number of omitted trials was averaged across signal duration (for signal trial omissions) and testing block, resulting in a single figure per rat for signal trial omissions, and a single figure per rat for non-signal trial omissions. Data were found to be non-normally distributed. Homogeneity of variance was tested using one-way ANOVA with absolute difference (the difference between the rank and the mean of rank for each data point) as the dependent and anaesthetic group as the factor. Group variances were found to be equal for both signal omissions and non-signal omissions (high event rate: both F values < 2.18; both p values > 0.132; short signal duration: both F values < 0.032; both p values > 0.99; predictable tone: both F values < 0.228; both p values > 0.978; predictable light distractor: both F values < 0.29; both p values > 0.75; irregular light distractor: both F values < 0.033; both p values > 0.98). As group variance were equal, data also satisfied the requirement of the Kruskal-Wallis test for similarly shaped distributions and therefore Kruskal-Wallis tests were used to analyse testing session omissions data. There were no group differences in signal trial omissions (high event rate: $H[2] = 0.492$, $p = 0.782$; short signal duration: $H[2] = 0.624$, $p = 0.732$;

predictable tone: H[2] = 0.078, p = 0.962; predictable light distractor: H[2] = 2.434, p = 0.296; irregular light distractor: H[2] = 0.624, p = 0.732) or non-signal trial omissions (high event rate: H[2] = 0.469, p = 0.791; short signal duration: H[2] = 0.36, p = 0.835; predictable tone: H[2] = 0.917, p = 0.632; predictable light distractor: H[2] = 0.363, p = 0.834; irregular light distractor: H[2] = 0.34, p = 0.835).

Experiment 2: effect of anaesthetic exposure during development on performance of the IDED during adulthood

Three performance measures were recorded: the number of trials to criterion, the time taken to make correct choices (correct choice latency) and the time taken to make incorrect choices (incorrect choice latency). The results of the IDED do not provide compelling evidence of an effect of developmental exposure to sevoflurane (either single or repeated exposure) on attention. An effect of prior anaesthetic exposure was detected on analysis of correct choice latency. However, this effect does not represent impaired performance of anaesthetised rats. A full description of the results is given below.

The number of trials to criterion

Trials to criterion data were analysed using repeated measures ANOVA with phase (7 levels: CD, SD, SDR, IDS, IDSR, EDS and EDSR) as the within-subjects factor and anaesthesia group (3 levels: group C [n=9], group 1A [n=9] and group 3A [n=7]) and sex (2 levels) as the between-subjects factors.

Exposure to anaesthesia during development did not alter the number of trials taken to reach criterion for the IDED task. As expected, the number of trials taken to reach criterion depended on the phase of the testing protocol ($F[6,114] = 5.7$, $p < 0.005$) but was not different between groups (main effect of group: $F[2,19] = 0.467$, $p = 0.634$) or between males and females (main effect of sex: $F[1,19] = 0.028$, $p = 0.868$) (see Fig 6.5). There was no phase by group interaction for trials to criterion ($F[12,114] = 0.533$, $p = 0.889$), no phase by sex interaction ($F[6,114] = 1.723$, $p = 0.122$) and no phase by group by sex interaction ($F[12,114] = 0.455$, $p = 0.936$).

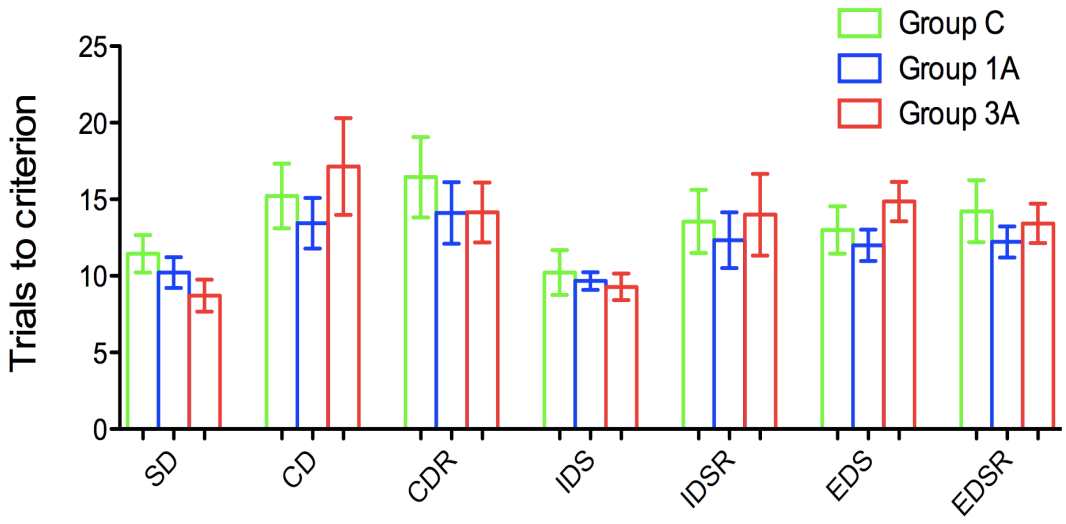

Figure 6.5 The number of trials taken to reach criterion during each of the seven testing phases. There was no group difference in the number of trials taken to reach criterion. Data are represented as mean +/- sem. SD: simple discrimination, CD: compound discrimination, CDR: compound discrimination reversal, IDS: inter dimensional shift, IDSR: inter dimensional shift reversal, EDS: extra dimensional shift, EDSR: extra dimensional shift reversal.

Although both the IDS and the EDS sessions represent new discrimination learning problems, the EDS requires shifting attention to a previously irrelevant dimension whereas the IDS does not. It is typically shown that more trials are taken to reach criterion during the EDS than the IDS, in order to support that the EDS requires this shift of attention. For this reason, a secondary repeated measures ANOVA was

constructed with phase (2 levels: IDS and EDS) as the within-subjects factor and group (3 levels: group C, group 1A and group 3A) and sex (2 levels) as between-subjects factors. All rats took more trials to reach criterion for the EDS (mean +/- sem 13.19 +/- 0.8) than the IDS (9.66 +/- 0.64) (main effect of phase: $F[1,19] = 24.72$, $p < 0.005$), and performance did not depend on either group or sex (all F values < 1.89, all p values > 0.178), confirming that all rats found the EDS more difficult than the IDS.

Correct choice latency

Correct choice latency data were analysed using repeated measures ANOVA with phase (7 levels: CD, SD, SDR, IDS, IDSR, EDS and EDSR) as the within-subjects factor and anaesthesia group (3 levels: group C [n=9], group 1A [n=9] and group 3A [n=7]) and sex (2 levels) as the between-subjects factors.

The effect of anaesthesia on the time taken to make a correct choice differed depending on whether rats were female or male and on which phase of the testing protocol they were completing, in other words there was an interaction between group, sex and phase for correct choice latency ($F[12,114] = 1.972$, $p = 0.033$).

This interaction was decomposed by examining the significance of the group by sex interaction for each one of three subsets of the phases: acquisition: SD and CD sessions where rats perform discriminations; set shift: IDS and EDS sessions where rats are required to shift attention to a new set of stimuli; and reversals: CDR, IDSR, and EDSR where rats are required to respond to previously unrewarded stimuli. Repeated measures ANOVAs were constructed with phase (2 levels for the acquisition and set shift subsets; 3 levels for the reversal subset) as the within-subjects factor and

anaesthesia group (3 levels: group C [n=9], group 1A [n=9] and group 3A [n=7]) and sex (2 levels) as the between-subjects factors. The effect of anaesthesia on performance during acquisition was dependent on whether rats were performing simple or compound discriminations and on whether they were female or male. Put another way, there was an interaction between phase (SD and CD), group and sex for the acquisition subset ($F[2,19] = 3.702$, $p = 0.044$) (Fig 6.6). Examination of the phase by sex interaction for each group separately revealed no significant effects (all F values < 4.71, all p values > 0.06). There were no other effects for the acquisition subset or for the set shift or reversal subsets (all F values < 2.96, all p values > 0.063).

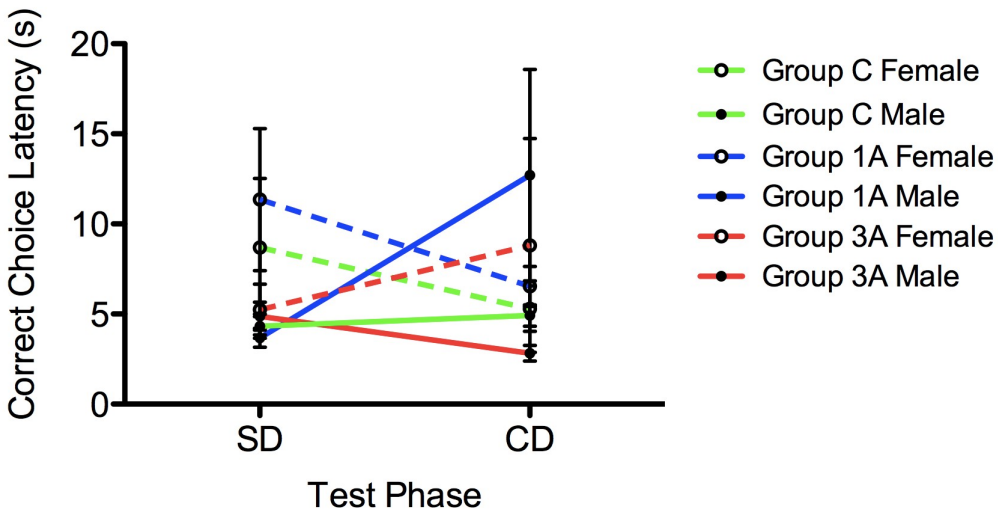

Figure 6.6 The time taken to make a correct choice (correct choice latency) during the simple discrimination (SD) and compound discrimination (CD) sessions. The effect of anaesthesia on performance during the two acquisition phases (SC and CD) was dependent on whether rats were performing simple or compound discriminations and whether they were female or male. Data are represented as mean +/- sem. SD: simple discrimination, CD: compound discrimination, CDR: compound discrimination reversal, IDS: inter dimensional shift, IDSR: inter dimensional shift reversal, EDS: extra dimensional shift, EDSR: extra dimensional shift reversal.

Incorrect choice latency

Some rats made no incorrect choices and so incorrect choice latency data were only analysed for the extra dimensional shift phase (EDS) where only one rat (a male from

group 3A) made zero incorrect choices i.e. where there was only one missing data point.

A one-way ANOVA with multiple factors (a univariate general linear model) was constructed with EDS as the dependent variable and group (3 levels: group C [n=9], group 1A [n=9] and group 3A [n=6]) and sex (2 levels) as the independent variables. Prior exposure to anaesthesia did not affect the time taken to make an incorrect choice during the extra dimensional shift testing phase. There was a trend towards an interaction of group and sex ($F[2,18] = 2.978$, $p = 0.076$) that did not reach statistical significance (Fig 6.7) and is therefore not considered further.

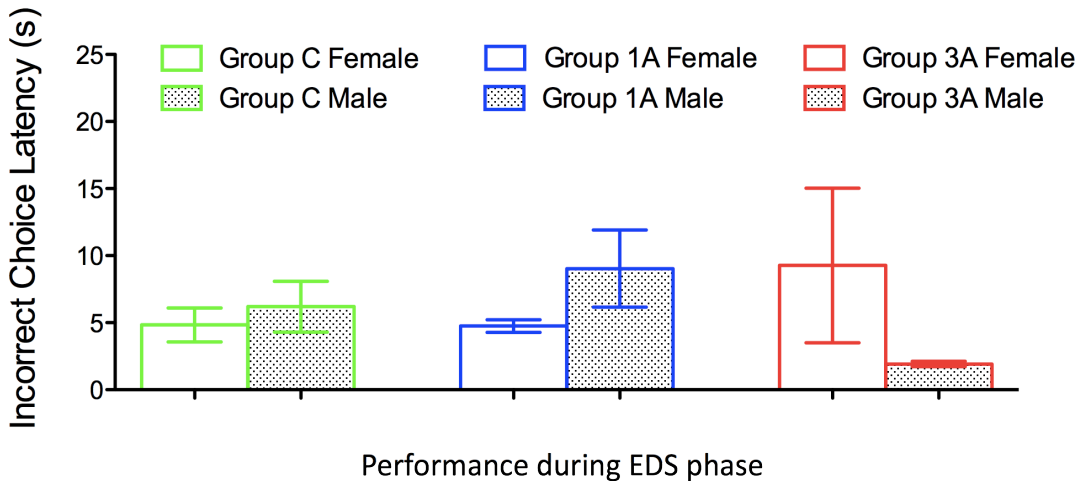

Fig 6.7 The time taken to make an incorrect choice (incorrect choice latency) during the extra dimensional shift (EDS) session. Prior exposure to anaesthesia did not affect the time taken to make an incorrect choice during the extra-dimensional shift testing phase. Data are represented as mean +/- sem.

Discussion

It was hypothesised that exposure to anaesthesia during development would lead to post-anaesthetic impairments in attention, and that the impairments would be greater in rats that had been repeatedly exposed to anaesthesia. Taken together the results of the SAT and IDED do not provide compelling evidence of an effect of developmental exposure to sevoflurane (either single or repeated) on attention.

Performance of the Sustained Attention Task was not impaired by anaesthetic exposure

Unfortunately, two of the task manipulations (increased event rate and predictable tone) did not appear to alter the difficulty of the task, and should be discounted when interpreting these results. Increasing the event rate of attentional tasks has been shown to increase task demands in humans (Parasuraman, 1979, Bushnell et al., 2003) and rats (McGaughy and Sarter, 1995, Bushnell, 1999). However, rats performing the high event rate task manipulation here did not demonstrate a decrease in their ability to detect or reject signals (there was no main effect of phase), nor did they demonstrate an increased vigilance decrement with increasing time on task (there was no interaction of phase and block). Therefore, the high event rate session here did not provide an increase in the attentional demands of the task, in other words rats did not find it more taxing than the baseline task, regardless of whether they had been exposed to anaesthesia during development. Similarly, during the predictable tone session there was no main effect of phase, and no interaction of phase with block or signal duration, demonstrating that this manipulation did not alter the demands of the task either.

Of the three SAT task manipulations where rats demonstrated either an increase (short duration session) or decrease (predictable and unpredictable light distractor sessions) in performance associated with the task manipulation, effects of prior anaesthetic exposure were also detected during two of these (the short duration and unpredictable light distractor sessions). Both effects were higher order interactions, that did not produce significant effects when statistically decomposed, perhaps suggesting only mild effects of exposure to sevoflurane on attentional processing, but should certainly be interpreted with caution.

In 2008 Newman and McGaughy found that adult rats that had received cholinergic lesions of the prefrontal cortex and those that received sham surgery 14 days prior to testing, demonstrated increased signal detection and a small decrease in signal rejection when signals of the shortest duration were presented alone rather than embedded within a dynamic stimulus range (Newman and McGaughy, 2008). This effect is thought to be due to the decrease in uncertainty associated with moving from a task manipulation that requires signal detection within a dynamic stimulus range and one that does not. This performance condition is hypothesised to be dependent on acetylcholine (Yu and Dayan, 2005, Newman and McGaughy, 2008). A similar pattern of increased signal detection apparently being offset by a temporary decrease in signal rejection was demonstrated by control rats here; however, rats that had been exposed to anaesthesia during development did not demonstrate the decrease in signal rejection. It is possible that this result indicates a difference in the strategy used to complete the task, but this is speculative and further work would need to be carried out to investigate this further.

As has been shown previously in both rats and humans (Newman and McGaughy, 2011, Demeter et al., 2011, Demeter et al., 2008), the unimodal (flashing light) distractor sessions impaired task performance in all rats. For the unpredictable light distractor session there was also a higher order interaction effect of prior exposure to anaesthesia on signal detection. However, this effect is difficult to interpret due to the lack of effects when the interaction was deconstructed.

Performance of the intra-dimensional extra-dimensional set shifting task was not impaired by anaesthetic exposure

Neither single, or repeated episodes of sevoflurane anaesthesia during development impaired performance of the IDED attentional set shifting task. There was a higher order interaction effect of prior exposure to anaesthesia on correct choice latency. This effect appeared to be driven by a sex difference in rats that had received one episode of anaesthesia during the acquisition phases, where male rats were slower than female rats to make correct choices during the simple discriminations and this sex difference was reversed during the compound discriminations. However, this effect does not represent impaired performance of rats that had received one episode of anaesthesia compared to controls. The possibility that rats could be detecting the odour of the food reward used for the IDED task cannot be excluded. Performance of the IDED task by control rats would normally be expected to be associated with reversal deficit, which does not appear to be the case here. An odour mask was used for the IDED task, with the aim of reducing this likelihood and data produced by control rats were similar to data previously collected in this laboratory where deficits on the task have been demonstrated (personal communication J McGaughy 2014). In addition, rats here demonstrated poorer performance during the ED phase than the

ID phase of the task, so performance cannot be entirely based on an ability to smell the food reward.

Could a transient effect of sevoflurane have been missed?

Volatile anaesthetic agents such as sevoflurane have a wide variety of ion channel targets. One of these targets is the N-methyl-D-aspartate (NMDA) receptor, where sevoflurane acts as an antagonist to down regulate excitatory signals (Campagna et al., 2003). Other anaesthetic agents that act more specifically as antagonists of NMDA receptors have been shown to cause impairment in attention. Adult rats given sub anaesthetic doses of ketamine demonstrate increased omissions when performing the SAT (Nelson et al., 2002) and increased trials to criterion when performing an extra-dimensional shift (Gastambide et al., 2013). However, these studies were designed to examine attentional performance soon after ketamine was administered to adult rats, i.e., before drug elimination was complete. In 2008 Broberg and colleagues investigated the effects of repeated administration of phencyclidine (PCP - an anaesthetic NMDA antagonist, uncommonly used in clinical settings) during development on IDED performance during adulthood (Broberg et al., 2008). On postnatal days 7, 9 and 13 male and female rats were given either vehicle, low or higher dose phencyclidine. Both male and female rats repeatedly exposed to PCP during development were found to be impaired at the extra-dimensional shift, with the effect in females being dose dependent. Here rats repeatedly exposed to sevoflurane during development were not impaired at the extra-dimensional shift, however they were tested at a later time point than rats in the Broberg study (P120+ as oppose to P53-93) and so it is possible that a more transient effect of sevoflurane was missed.

How do the results relate to those of experiments within previous chapters?

The age of onset of behavioural testing chosen for the study presented here (P91 - P120), was guided by previous work demonstrating spatial working memory impairments in three-month-old rats that had been exposed to isoflurane during development (Chapter 5). However, a direct comparison of the effects of anaesthetic exposure during development, on spatial memory (Chapter 5) and attentional performance is limited because a different anaesthetic regimen was used in each of the chapters (isoflurane and nitrous oxide in Chapter 3 and sevoflurane in the absence of nitrous oxide here). Agent dependent effects of anaesthetic-induced neurotoxicity have been demonstrated. Post exposure neurotoxicity in exvivo neurons, pre-injured by oxygen-glucose deprivation, has been demonstrated with isoflurane but not sevoflurane or desflurane (Schallner et al., 2014). In addition, exvivo rat cortical neurons exposed to isoflurane but not sevoflurane demonstrate increased cytostolic calcium concentration and resulting cytotoxicity (Wang et al., 2008). However importantly, an invivo study conducted by Istaphanous and colleagues found that neonatal mice exposed to equipotent anesthetic concentrations of isoflurane, sevoflurane or desflurane at P7, demonstrated similar levels of anaesthetic- induced neuronal apoptosis. Collectively these results present an unclear picture of potential agent dependent effects of anaesthesia, however, they suggest that the change in anaesthetic regimen between Chapters 3 and 5 here limits comparison of results. In explanation, the decision to change the anaesthetic regimen between chapters was a result of the length of time taken to carry out experiments. It was driven by two factors (i) as time passed the field of anaesthetic-induced neurotoxicity moved away from the use of nitrous oxide, with an increased focus on single agents; (ii) an

increasing concern within the field, that rodent studies would benefit from greater continuity with non-human primate studies, in order to better understand the translatability of results. The anaesthetic regimen chosen here was therefore chosen to be most similar to that used in the non-human primate studies conducted by Raper and colleagues (Raper et al., 2016, Raper et al., 2015).

Summary

Overall the results of this study do not provide evidence for dramatic anaesthetic-induced impairments in attention for adult rats exposed during development. The absence of anaesthetic effects on attention here, contrasts the sustained impairments in learning and memory resulting from developmental exposure, of rodents, to a range of anaesthetic agents (Chapter 5, (Jevtovic-Todorovic et al., 2003b, Fredriksson et al., 2007)), including sevoflurane. This perhaps suggests that the phenotype of developmental anaesthetic-induced cognitive impairment does not involve disruptions to attentional processing; however, further work is needed to determine if this is in fact the case.

CHAPTER 7: THE EFFECT OF EXPOSURE TO ISOFLURANE PLUS NITROUS OXIDE ANAESTHESIA DURING ADULTHOOD ON SYNAPTIC INTEGRITY

Introduction

The neuronal mechanisms underpinning anaesthetic-induced cognitive impairment are unclear, and may differ depending on the age at which anaesthetic exposure occurs. The peak of synaptogenesis in rodents occurs at around postnatal day (P) 7 and this developmental time point appears to be a period of vulnerability to the toxic effects of anaesthetic agents (Yon et al., 2005). Anaesthetic exposure at this developmental time point commonly leads to neuronal apoptosis (Satomoto et al., 2009, Jevtovic- Todorovic et al., 2003b, Head et al., 2009); a process that occurs throughout both cortical and subcortical brain areas (Jevtovic-Todorovic et al., 2003b), via both the intrinsic and extrinsic apoptotic pathways (Yon et al., 2005), and has been associated with the induction of cognitive impairment (Jevtovic-Todorovic et al., 2013).

However, anaesthetic exposure outside of this period of vulnerability is not invariably accompanied by apoptosis, suggesting that there may be other potential mechanisms for anaesthetic-induced cognitive impairment. Rats or mice, exposed repeatedly to isoflurane anaesthesia, at either P14 or P60 fail to demonstrate neuronal apoptosis of hippocampal neurons. Those exposed at P14 demonstrate an impairment in an object

recognition task (the only behavioural testing conducted) (Zhu et al., 2010); although it is important to note that the hippocampal dependency of object recognition is controversial and still being debated. The same study found that cognitive impairment in the developmentally exposed rodents, but not those exposed during adulthood, was associated with decreases in hippocampal neurogenesis and in the hippocampal stem cell pool, so it is certainly not the case that the hippocampus is resistant to the toxic effects of isoflurane. The lack of anaesthetic-induced apoptosis in this study demonstrates that it may not be a necessary consequence of anaesthetic exposure.

It is not just gross cellular events (apoptosis and reduced neurogenesis) that occur as a result of anaesthetic exposure; sub cellular changes also occur that could lead to changes in neuronal plasticity. Rats exposed on a single occasion at P16, to any one of three commonly used volatile anaesthetic agents (isoflurane, sevoflurane and desflurane), demonstrate morphological changes to the ultrastructure of neurons of the medial prefrontal cortex (Briner et al., 2010). The anaesthetic agents did not cause apoptosis but rather an increase in the density of dendritic spines on the apical and basal dendrites. The duration of anaesthesia required to produce these effects varied depending on the agent, suggesting that the increase in dendritic spine density was not simply a function of the altered state of consciousness and necessary sensory deprivation, but was at least partly dependent on the direct effects of the drugs themselves.

Exposure to alcohol might be expected to cause similar toxic changes as exposure to volatile anaesthesia, given the structural similarity between the drugs. However, rodents exposed chronically to ethanol demonstrate a loss of (rather than increase in)

dendritic spines (Tavares et al., 1983, Lescaudron et al., 1989). These sub cellular changes occur in the absence of the profound loss of sensory input associated with the state of anaesthetic unconsciousness, again providing support for the view that anaesthetic-induced changes to neuronal ultrastructure are not dependent on the state of anaesthesia per se but rather exposure to anaesthetic drugs. There are many differences between these studies that could account for the difference in spine density response - the life stage at exposure or acute versus chronic exposure being particularly important variables.

Dendritic spines are dynamic structures that can undergo rapid morphological change. The rapidity with which structural changes occur is thought to be an essential component of short and long-term functional plasticity (Kasai et al., 2010). It has been suggested that the frequent spine generation and elimination that occurs in the adult neocortex is the mechanism by which stable memories are formed (Kasai et al., 2010, Yang et al., 2009). In support of the importance of spine dynamics for cognitive function, neurological pathologies involving cognitive impairment are associated with alterations in dendritic morphology or spine density. Human patients suffering from chronic alcoholism, dementia, Alzheimers disease and schizophrenia have a loss of dendritic spines or gross distortion of spine structure (Ferrer et al., 1986, Catala et al., 1988, Akram et al., 2008, Garey et al., 1998). These rapid changes in dendritic form are facilitated by an actin-based cytoskeleton (Sorra and Harris, 2000), the organisation of which can be altered by exposure to volatile anaesthetic agents (Kaech et al., 1999), thereby providing a potential mechanism by which anaesthesia could cause structural changes to dendritic spines.

Most excitatory neuronal connections are made at the head of each dendritic spine. Spine density measurement can therefore be used to estimate excitatory synapse density, which may then provide insights into functional ability or plasticity (Holtmaat and Svoboda, 2009). Although spine morphology is thought to be a continuum from short, thin, headless protrusions to large or long, thick necked, pedunculated appendages, spine morphologies may be classified into different types in order to facilitate determining shifts in morphology that may occur with experimental manipulations (Sorra and Harris, 2000, Dickstein et al., 2010, Radley et al., 2006, Kirov et al., 1999).

In Chapter 3 rats exposed to 2 hours of isoflurane and nitrous oxide anaesthesia demonstrated a deficit in a hippocampal dependent task of spatial memory, when testing began 48 hours after anaesthetic exposure. In the current study, the effect of 2 hours of the same anaesthetic regimen (isoflurane plus nitrous oxide) on Cornu Ammonis 1 (CA1) hippocampal cell morphology, overall dendritic spine density, and the spine density of three predetermined morphologically classified spine types (thin, stubby and mushroom spines), was examined. Anaesthetic exposure occurred during adulthood, at the same age as for rats in Chapter 3, in order to facilitate comparison of histological results with the behavioural results found in Chapter 3. Intracellular iontophoretic injections of Lucifer Yellow were made into CA1 hippocampal cells from rats euthanised 48 hours following anaesthetic exposure. Confocal laser scanning microscopy was carried out on systematic-randomly chosen dendritic segments at a set radial distance from the cell soma, and validated computer software used to reconstruct three dimensional images for analysis. This methodology has been shown to produce results comparable with results from serial section transmission electron microscopy but in a fraction of the time and with better

preservation of the tissues (for a methods paper see (Dumitriu et al., 2011)). It was hypothesised that anaesthetic exposure would be associated with a change in dendritic spine density - either an increase (as seen in studies of anaesthetic exposure during development), or a decrease in spine density (as seen in chronic exposure to alcohol and some other pathological conditions).

Materials and Methods

Subjects

Male Long Evans rats (Taconic, USA), 4 months old (323-401g) at the time of anaesthesia were pair (or triple) housed, in polycarbonate cages with automatically regulated lighting (12/12h light/dark cycle, lights on at 2000h). Rats were given ad libtum access to food and water.

Experimental design

Rats were randomly allocated to one of two groups : control condition (n=4) or one 2 hour period of anaesthesia (n=5), counterbalanced for body weight. In line with the principle of reduction, rats used were 'spare' naive rats from an unrelated experiment, hence the uneven group numbers. A recovery period of 48 hours after cessation of anaesthetic, or of cessation of the control condition, was used to ensure that the anaesthetic had been eliminated from the brain and to parallel the experimental design used in Chapter 3. Forty-eight hours after anaesthesia rats were terminally anaesthetised and transcardially perfused with paraformaldehyde in order to fix the brain tissue for histological analysis. The brain tissues were extracted, processed and stored (see below for details) before dendritic ultrastructure was determined. Six neurons were examined per rat.

Anaesthesia and control condition

Episodes of anaesthesia consisted of 2 hours of 1.2% isoflurane plus 70% nitrous oxide, 30% oxygen. Rectal temperature was maintained at 37C +/- 0.5C. Indirect blood pressure was recorded at the end of each anaesthetic hour (i.e., 2 samples per animal). In addition, a venous blood sample was taken at the end of anaesthesia, and immediately analysed for pH, pCO_2 and pO_2. The control condition consisted of 2 hours of medical air plus oxygen (fraction of inspired oxygen 0.3), in an identical chamber with identical gas flow rate (see General Methods (Chapter 2) Section 4 for more details of conduct and monitoring). Following emergence from anaesthesia, all rats were returned to their home cages where they remained for 48 hours before being terminally anaesthetised and transcardially perfused.

Stereology

Tissue preparation

Forty-eight hours after exposure to anaesthesia or the control condition, rats were anaesthetised with intra-peritoneal pentobarbitone (100mg/kg) and transcardially perfused, at a rate of 25 ml/min, with ice cold 1% paraformaldehyde in 0.1 molar phosphate-buffered saline (PBS) (pH 7.4) for three minutes; followed by fixation with 4% paraformaldehyde in 0.1 molar phosphate-buffered saline (PBS) (pH 7.4) for 15 minutes at a rate of 25 ml/min.

The brains were carefully dissected and postfixed overnight at 4°C in 4% paraformaldehyde plus 0.125% gluteraldehyde in PBS, before being cut into 250

micrometer coronal sections on a Vibratome (Leica VT1000S, USA) and stored at 4°C in PBS with 0.1% sodium azide, until required for imaging.

Intracellular Dye Injections

On the day of injection, brain slice sections were passively warmed to room temperature and were then incubated in 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 5 minutes, to reveal the cytoarchitectural features of the pyramidal layer of CA1 in the hippocampus. The sections were then mounted on nitrocellulose paper and immersed in ice-cold 0.1 molar PBS. Using light microscopy, pyramidal neurons of the CA1 layer were impaled with a sharp glass micropipette and loaded with 5% Lucifer Yellow in distilled water under a DC current of 3 - 8 nA for 5 – 10 minutes, or until dye had completely filled distal processes and no further loading was observed. Loaded neurons (6 per slice) were chosen to be far enough apart to avoid overlapping of their dendritic trees. The sections where then mounted on gelatin-coated glass slides and coverslipped in Fluoromount G (Beckman Coulter, Fullerton, CA, USA). Six neurons (3 per hemisphere) per animal were included in the analysis. In order for a loaded neuron to be included in the analysis it had to lie within the CA1 region of the hippocampus, demonstrate complete filling of the dendritic tree, demonstrate intact primary and secondary branches and demonstrate intact tertiary branches (with the exception of branches that extended beyond 50 micrometers in radial distance from the cell soma).

Neuronal mapping

Neuronal cell mapping was carried out to enable the examination of dendritic arborisation. Images were captured using a 2.5 x objective with a 0.75 numerical

aperture and an oil emersion 40 x objective with a 1.3 numerical aperture, on an Axiophot-2 microscope (Zeiss, NY, USA). The microscope consisted of a motorised stage, a video camera system and Neurolucida morphometry software (MBF Bioscience, Williston, VT, USA). The system allows for accurate tracing of cell processes in three dimensions (X, Y and Z planes).

The Neurolucida morphometry software allows the user to trace a displayed image whilst moving the microscope stage in 1-micrometer steps through the Z-axis, resulting in dendrograms for each neuron, which show the dendritic branching pattern. Using NeuroExplorer software (MBF Bioscience) the total dendritic length (the summed length of dendritic segments for either the apical or basal neuronal tree) were calculated for each neuron. Concentric circles were then drawn on the dendrograms at 30 micrometer increments from the soma. These were used to determine the number of intersections and the amount of dentritic material per radial distance from the soma - a process called Sholl analysis (Sholl, 1953).

Confocal laser scanning microscopy and spine acquisition

The confocal laser scanning microscopy method used for morphometric analysis of dendritic spines was as in Dumitriu et al., 2011. This stage of imaging involved the use of a confocal laser scanning microscope (Zeiss LSM 510, NY, USA), equipped with a 458 nanometer excitation wavelength, 30 milliwatt power and 6 ampere tube current, argon laser; a 100 x objective (Zeiss Apochromat) with a 1.4 numerical aperture and a digital zoom. As the density of dendritic spines varies with dendrite circumference, which in turn varies with distance from the soma, dendritic segments of 20-25 micro meters in length were selected for imaging by drawing a circle at 100

micro meters from the cell body, and imaging dendritic segments that intersected with the circle (as in (Radley et al., 2006, Dickstein et al., 2010, Midthune et al., 2012) (Fig 7.1).

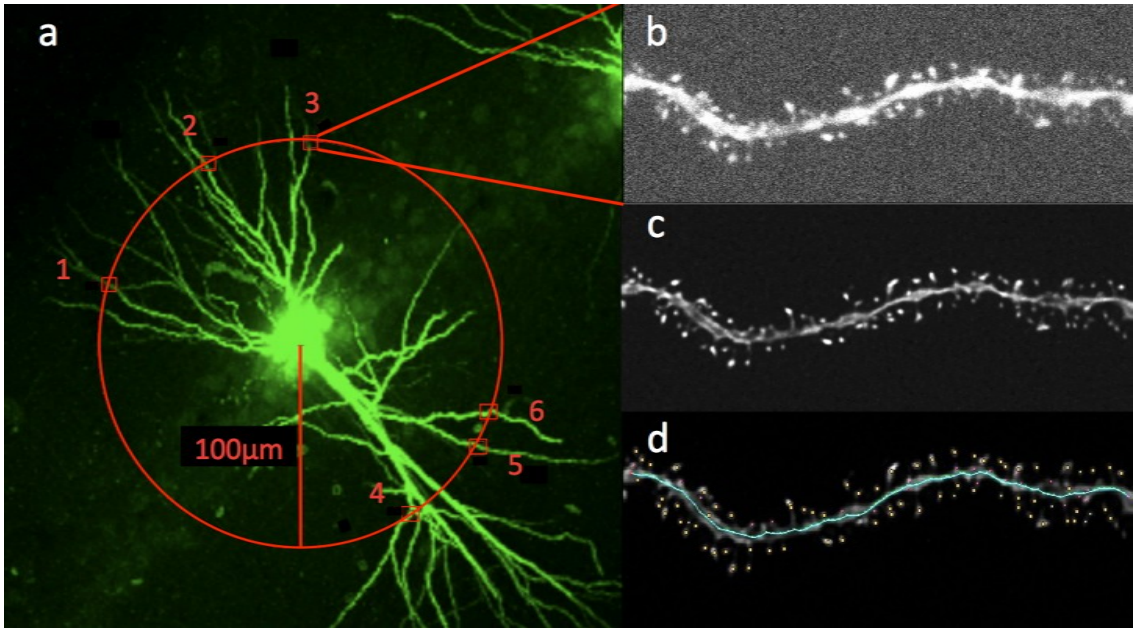

Figure 7.1 (a) CA1 hippocampal neuron after intra-cellular dye injection with lucifer yellow and viewed at 40 x magnification. A red circle with a radius of 100µm marks the distance from the cell soma, at which dendritic segments (labelled 1-6) were imaged. (b) Dendritic segment 3 after confocal laser scanning microscopy. (c) Dendritic segment 3 after deconvolution of the image to reduce optical aberration. (d) Dendritic segment 3 after automated spine classification.

In order for a dendritic segment to be optically imaged it had to satisfy the following criteria: (i) the entire segment had to fall within a depth of 50 micro meters, (ii) dendritic segments had to be either parallel or at acute angles to the coronal surface of the section, (iii) segments did not overlap other segments that would obscure visualization of spines, (iv) segments were not within the first or last 10% of the dendritic length and all segments occurred at approximately the same distance along the dendrite (Radley et al., 2006, Dickstein et al., 2010, Dumitriu et al., 2011). Use of these criteria was aimed at reducing sampling bias. Three dendritic segments were imaged per apical and basal neuronal tree (6 segments per neuron). Serial 2-

dimensional images (X by Y axis) were taken of each segment at different depths (Z axis), to form stacks of images that were used to reconstruct a 3 dimensional image of the segment (a confocal stack). All confocal stacks were acquired at 512 x 512 pixel resolution with a Z axis-step of 0.1 micrometers, a pinhole setting of 1 Airy Unit (a measure of pin hole diameter) and optimal settings for gain and offset, and included approximately 1 micrometer above and below the identified dendritic segment.

To improve voxel resolution and reduce optical aberration along the Z-axis, the acquired images were deconvolved using an interactive blind deconvolution algorithm (AutoDeblur version 8.0.2; MediaCybernetics, Bethesda, MD, USA). This step enables precise interpretation of 3-dimensional spine morphology, that would be otherwise blurred due to image spread in the Z plane (Rodriguez et al., 2008, Rodriguez et al., 2006).

Spine analysis

After deconvolution, the confocal images were analyzed using NeuronStudio software (Computational-Neurobiology-Imaging-Centre, 2015) to examine global and local morphometric characteristics of dendrites and spines such as dendritic spine densities and dendritic spine shape (stubby, mushroom, and thin). This software allows for automated digitisation and reconstructions of 3-dimensional neuronal morphology from multiple confocal stacks on a spatial scale, and averts the subjective errors encountered during manual tracing, through use of a Rayburst-based sampling algorithm (for method development see: (Rodriguez et al., 2006, Rodriguez et al., 2008). The 3-dimensional reconstructions were displayed (see Fig 7.1), each one

inspected manually for accuracy and appropriate corrections made before automated spine classification. Spines were classified into 3 categories according to their shape (thin, stubby or mushroom). A spine was labeled thin or mushroom if the ratio of their maximum head diameter to maximum neck diameter was more than 1.1:1. If spines that fit those criteria additionally had a maximum head diameter of less than 0.35 micrometers, they were classified as thin spines, and if the head diameter was bigger than that, they were considered mushroom spines. In order to be classified as stubby spines, the head to neck ratio had to be smaller than 1.1:1.

Unfortunately, due to a methodological error, the mounting of slides from two rats in the anaesthesia group deteriorated after neuronal mapping (and was not rescued by remounting), such that they could not be used for dendritic spine analysis. Group numbers for the spine analysis were therefore, control condition (n=4) and one 2-hour period of anaesthesia (n=3).

Statistical Analysis

Anaesthetic physiology

Blood pressure data from anaesthetised rats were compared using repeated measures ANOVA, with sample (2 levels: 1hr and 2hr post-induction of anaesthesia) as the within-subjects factor. All data are presented as mean +/- SEM.

Dendritic morphology

Data for apical and basal dendritic trees were analysed separately. Data were averaged across neurons to provide a single figure (mean value) per animal. The total

dendritic length for each each group (anaesthesia or control) was compared using independent two-tailed students t-tests. The dendritic length per radial distance from the soma, and separately, the number of intersections per radial distance from the soma, for each group were compared using repeated measures ANOVA with radial distance from the soma (15 levels for apical dendrites: 30 μm increments from the soma; 8 levels for basal dendrites: 30 μm increments from the soma) as the within-subjects factor and anaesthesia group (2 levels) as between-subjects factor.

Spine morphology

Data for apical and basal dendritic segments were analysed separately. Data were averaged to provide a single figure (mean value) per animal for total spine density, and three figures (mean values) per animal for the density of each spine type (thin, stubby and mushroom shaped spines), separately for apical and basal dendrites. The total dendritic spine density data were compared (anaesthesia versus control) using independent two-tailed students t-tests. The densities for the three spine types were compared using repeated measures ANOVA with spine type (3 levels: thin, stubby, mushroom) as the within-subjects factor and anaesthesia group (2 levels) as the between-subjects factor.

Results

Anaesthetic physiology

Body temperature was successfully maintained between 36.5C and 37.5C in all anaesthetised rats.

Blood pressure and blood gas parameters remained within the normal physiological range during anaesthesia. As expected, blood pressure values were slightly lower than reported values in non-anaesthetised rats (Table 7.1).

Parameter	**Min – Max**	**Mean ± SEM**	**Non-anaesthetised values (Culley et al., 2003)**
Mean arterial blood pressure 1hr after induction of anaesthesia (mmHg) (n=4)	87 -118	102 ± 4	113 ± 3
Mean arterial blood pressure 2hr after induction of anaesthesia (mmHg) (n=4)	94- 109	100 ± 3	113 ±3
pH (n=4)	7.4 -7.49	7.43 ± 0.02	7.45 ± 0.02
pO2 (n=4)	84 – 156	108 ± 11	84 ± 6
pCO2 (n=4)	37 – 49	43.4 ± 1.8	36 ± 1

Table 7.1: Blood pressure and blood gas parameters during anaesthesia remained within the normal physiological range. Rats received 2 hours of anaesthesia, with 1.2% isoflurane and 70% nitrous oxide, 48 hours prior to euthanasia and brain tissue collection. Blood pressure readings were taken every hour. Blood gas samples were taken at the end of the 2hr period of anaesthesia. Non-anaesthetised values are provided only for interest and are taken from (Culley et al., 2003a). Data are presented as mean +/- SEM.

Dendritic morphology

Dendritic length

This parameter was assessed in a total of 9 subjects. Prior anaesthetic exposure did not affect total dendritic length for apical or basal dendrites. The total dendritic length for control rats was 3228.4 +/- 152.9 μm (mean +/- SEM) for apical dendrites and 617.2 +/- 57.0 μm for basal dendrites; as compared to 3554.2 +/- 360.0 μm for apical dendrites from anaesthetised rats and 644.3 +/- 47.1 μm for basal dendrites from anaesthetised rats. The dendritic length of apical and basal dendrites was unaffected by anaesthetic exposure (apical: $t(7) = -0.758$, $p = 0.47$; basal: $t(7) = -0.371$, $p = 0.72$) (Fig 7.2).

Sholl analysis

This parameter was assessed in a total of 9 subjects. There were no group differences in either dendritic length per radial distance from the soma or the number of intersections per radial distance from the soma, for apical or basal dendrites. There were no main effects of group for dendritic length per radial distance from the soma, for apical ($F[1,7] = 0.606$, $p = 0.46$) or basal dendrites ($F[1,7] = 0.482$, $p = 0.51$). As expected, there were main effects of distance from the soma (apical: $F[14,98] = 83.61$, $p < 0.005$; basal: $F[7,49] = 81.521$, $p < 0.005$) but these did not interact with group (apical: $F[14,98] = 0.956$, $p = 0.50$; basal: $F[7,49] = 0.236$, $p = 0.97$). Similarly, there were no main effects of group for the number of intersections per radial distance from the soma, for apical ($F[1,7] = 0.542$, $p = 0.49$) or basal dendrites ($F[1,7] = 0.857$, $p = 0.39$). Again as expected, there were main effects of distance from the soma (apical: $F[14,98] = 91.194$, $p < 0.005$; basal: $F[7,49] = 91.685$, $p < 0.005$) but these did not

interact with group (apical: F[14,98] = 0.721, p = 0.75; basal: F[7,49] = 0.436, p = 0.88) (Fig 7.2).

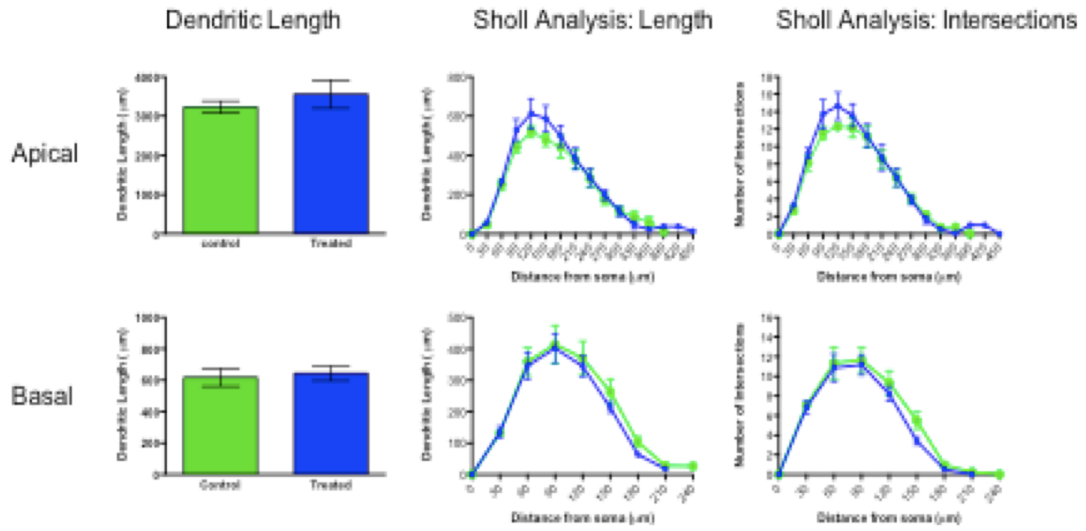

Figure 7.2: Neuronal mapping data for apical (upper 3 subfigures) and basal (lower 3 subfigures) dendritic arbors of CA1 hippocampal cells, from rats that received 2 hours of 1.2% isoflurane and 70% nitrous oxide (blue) or rats exposed to control conditions (30% oxygen) (green).

Spine morphology

Total spine density

This parameter was assessed in 7 subjects. The total spine density was calculated to give a single figure per rat, by averaging across dendritic sections. The average spine density for neurons from control rats was 3.36 +/- 0.16 µm (mean +/- SEM) for apical dendrites and 3.03 +/- 0.24 µm for basal dendrites; as compared to 3.68 +/- 0.32 µm for apical dendrites from anaesthetised rats and 3.71 +/- 0.2 µm for basal dendrites from anaesthetised rats. Exposure to anaesthesia did not affect the total spine density on apical dendrites ($t(5) = -1.291$, $p = 0.25$). There was a trend towards an increase in spine density on basal dendrites from anaesthetised rats but this did not reach

statistical significance ($t(5) = -2.417$, $p = 0.06$) (Fig 7.3), suggesting that this study was underpowered.

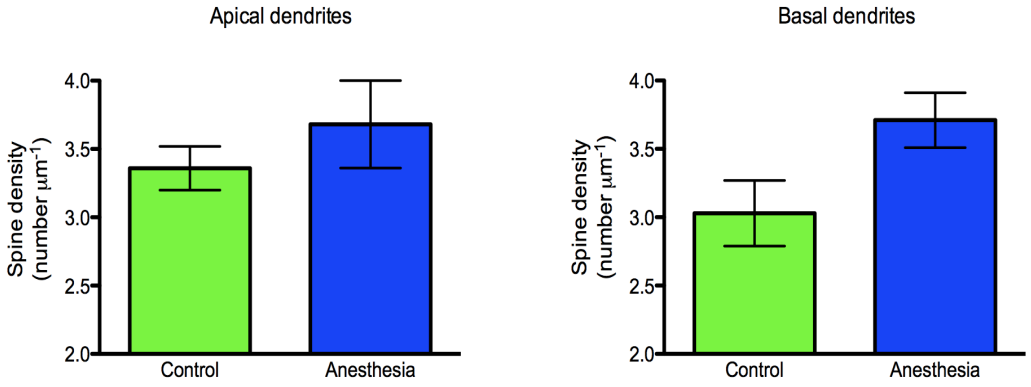

Figure 7.3: The total spine density (number of spines per µm of dendritic length) for rats that received 2 hours of 1.2% isoflurane and 70% nitrous oxide (blue) or rats exposed to control conditions (30% oxygen) (green), for apical dendrites (left subfigure) and basal dendrites (right subfigure). Exposure to anaesthesia did not alter total spine density on apical or basal dendrites.

Spine type

This parameter was assessed in a total of 7 subjects. Exposure to anaesthesia did not affect spine type density differentially. For the average spine density of each of the three spine types, on both apical and basal dendrites, see Table 7.2.

	Apical dendrites	**Basal dendrites**				
	Thin spine density (number per µm)	**Stubby spine density (number per µm)**	**Mushroom spine density (number per µm)**	**Thin spine density (number per µm)**	**Stubby spine density (number per µm)**	**Mushroom spine density (number per µm)**
Control rats	2.15 +/- 0.22	1.08 +/- 0.04	0.11 +/- 0.02	1.89 +/- 0.24	1.07 +/- 0.02	0.06 +/- 0.02
Anaesthetised rats	2.37 +/- 0.15	1.19 +/- 0.04	0.08 +/- 0.02	2.47 +/- 0.09	1.2 +/- 0.08	0.04 +/- 0.01

Table 7.2: The spine density (number of spines per µm of dendritic length) for each of the three spine types (thin, stubby and mushroom) for rats exposed to control conditions (30% oxygen) or rats that received 2 hours of 1.2% isoflurane and 70% nitrous oxide. Data presented as mean +/- SEM. Exposure to anaesthesia did not affect spine type differentially.

As expected from the results of total spine density (above), there was no main effect of group for either apical ($F[1,5] = 1.507$, $p = 0.27$) or basal ($F[1,5] = 5.592$, $p = 0.06$) dendrites, although anaesthetic exposure did induce an increase in spine density on basal dendrites that did not reach statistical significance. As expected, there were main effects of spine type for both apical ($F[2,10] = 135.74$, $p < 0.005$) and basal ($F[2,10] = 137.56$, $p < 0.005$) dendrites, with thin spines predominating and the lowest density being mushroom spines. Anaesthesia was associated with numerical increases in thin and stubby spines and a decrease in mushroom spines but these changes did not reach statistical significance - there was no interaction between group and spine type for either apical ($F[2,10] = 0.456$, $p = 0.65$) or basal ($F[2,10] = 2.997$, $p = 0.09$) dendrites. (Fig 7.4).

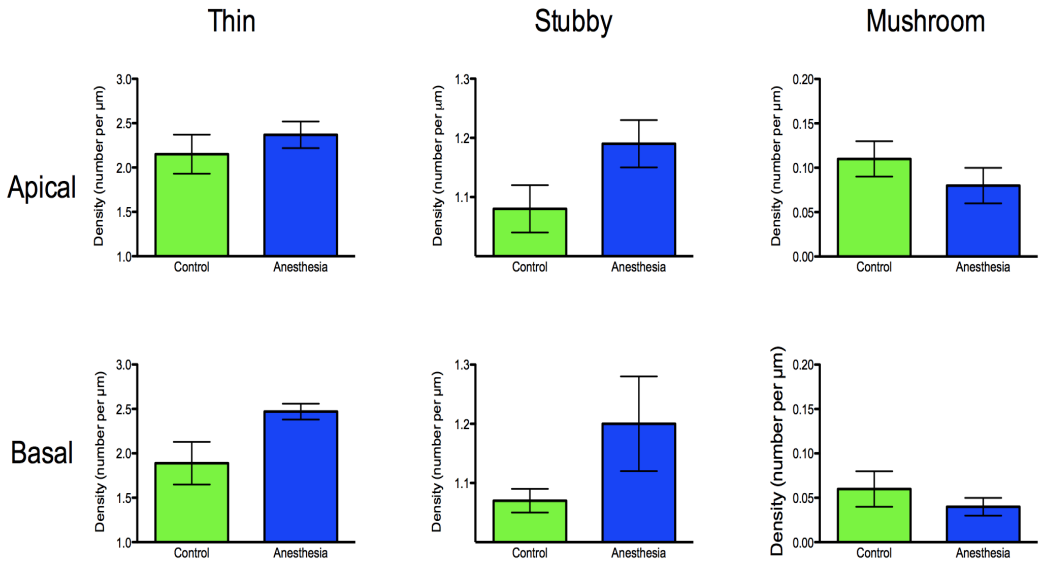

Figure 7.4: Total spine density for each spine type (thin, stubby and mushroom spines), on apical (upper 3 subfigures) or basal (lower 3 subfigures) dendrites, for rats that received 2 hours of 1.2% isoflurane and 70% nitrous oxide (blue) or rats exposed to control conditions (30% oxygen) (green). Exposure to anaesthesia did not affect the density of different spine types. Note that Y axis scales are different for each spine type.

Discussion

Exposure to 2 hours of isoflurane and nitrous oxide anaesthesia did not alter the total dendritic length or arborisation of CA1 hippocampal cells. Anaesthetic exposure also did not alter the density of spines on apical or basal dendrites, however it was associated with an increase in spines on basal dendrites that did not quite reach statistical significance.

Results from the control group are similar to pre-established values

The results obtained from non-anaesthetised rats in this study are similar to those of other studies, examining results in control animals, supporting the reliability of the results. McMullen et al used golgi cox staining to examine CA1 hippocampal cell arborisation from adult Long Evans rats. When conducting Sholl analysis, they found that the maximum mean number of intersections was approximately 13, and occurred approximately 70 μm from the cell soma (McMullen et al., 1984). In the study here results were presented separately for apical and basal dendrites, but are similar nonetheless (maximum mean number of intersections = 12, occurring at 120 μm on the apical dendritic arbour and 60 μm on the basal dendritic arbour). In addition, the total spine density of control rats found here (3 − 3.3 spines per μm) fits within the range reported by Dumitriu et. al., who found that the spine density of CA1 hippocampal cell dendritic segments from control male rats ranged from 2.76 to 6.83 spines per μm (Dumitriu et al., 2011). Using confocal laser scanning microscopy and NeuronStudio software i.e., the same methodology used here, they found the mean spine density was 4.33 spines per μm. This value is slightly higher than that found here, however it is of note that they sampled dendritic segments at a different distance from the cell some

(150-200 μm), as oppose to 100 μm. The resulting difference in diameter of dendritic segments sampled and therefore spine density of those segments could account for the slight discrepancy (Dumitriu et al., 2011).

Anaesthetic exposure did not affect the dendritic ultrastructure of adult rats

Exposure to the volatile and gaseous anaesthetic agents isoflurane and nitrous oxide, during adulthood, did not result in changes to the dendritic ultrastructure of adult rats. This result is in line to that of a study that examined the effects of exposure to injectable anaesthetic agents on the CA1 hippocampal dendritic ultrastructure of adult mice. De Roo and colleagues exposed mice at P30 to one of three injectable anaesthetic agents (midazolam, ketamine or propofol). Mice received a 5-hour exposure to anaesthesia and were then euthanised and brain tissue examined using confocal laser microscopy. No difference in dendritic spine density was found between any of the anaesthesia groups and control animals (De Roo et al., 2009). However the same study found that an increase in dendritic spine density was found when anaesthetic exposure occurred at either P15 or P20, suggesting that the effects of anaesthesia on dendritic ultrastructure are restricted to earlier stages of development. In support of this view are the findings by Huang & Yang who examined the effects of either a single exposure or repeated exposures to a combination of the injectable anaesthetic agents ketamine and xylazine (Huang and Yang, 2015) on mice. With the use of repeat (pre and post training) in-vivo imaging of fluorescently labelled postsynaptic dendritic spines in the motor cortex, the authors found that learning-dependent spine plasticity in the primary motor cortex was decreased when mice were exposed to repeated (but not single) episodes of anaesthesia at P14-P18, but not when

exposure occurred at a later developmental time point (P21-P25). The changes to dendritic ultrastructure were associated with impaired motor learning, as assessed by a rotarod task, with behavioural and histological analyses being carried out when animals were P30.

Limitations

Taken together these results suggest that, regardless of the type of anaesthetic agent (volatile or injectable agents), anaesthetic exposure in adulthood does not lead to changes in dendritic ultrastructure. However, the results presented here are not entirely conclusive. The loss of spine analysis samples (2 rats) from the group of rats that received anaesthesia was unfortunate, and compromised the projected number of samples planned for the experiment, resulting in a loss of actual statistical power compared to intended statistical power. Unfortunately, this means that morphological changes as a result of exposure to isoflurane and nitrous oxide during adulthood cannot be entirely ruled out, as there was an increase in spine density that did not reach statistical significance, supporting the view that the study was underpowered. Such morphological changes could potentially be an underpinning mechanism for anaesthetic-induced cognitive impairment, but further investigation is needed and unfortunately considerations of time and resources prevented pursuing this within the context of the thesis. Future experiments could investigate the effect of isoflurane and nitrous oxide on spine morphology within a design that might be expected to enhance affects, e.g., repeated short exposures. Negative results in such a context would enable a more definitive conclusion to be made.

CHAPTER 8: GENERAL DISCUSSION

Overall summary of results

Aim 1: To better understand the behavioural phenotype of anaesthetic-induced cognitive impairment

In order to provide an anchor point for this work and build on existing literature, the previously reported effect of anaesthesia on win-shift spatial memory (Culley et al., 2004b), was reproduced in Chapter 3. Results were extended by demonstrating that the anaesthetic-induced impairment in win-shift spatial memory was not associated with impairment in novelty preference (Chapter 3), or aspects of attention (Chapter 4) - both of which are candidates for cognitive processes involved in successful completion of the win-shift radial arm maze task (Sanderson et al., 2007, Sanderson and Bannerman, 2012). Mild anaesthetic-induced impairment *was* seen in the 5CSRTT, following 2hrs of isoflurane plus nitrous oxide anaesthesia. However, in marked contrast to win-shift radial maze performance, impairment was not seen in multiple performance measures and did not increase in magnitude with repeated exposure (see Aim 2). It is therefore seemingly unlikely that this attentional impairment is sufficient to be entirely responsible for the radial maze impairment detected.

It should be noted that a multiple anaesthetic exposure group was not included in the assessment of spontaneous, spatial, novelty preference, in rats exposed in adulthood. An effect of multiple, but not single, anaesthetic exposures was found when examining

spatial memory (on the radial arm maze) in neonates (Chapter 5). It is, therefore, possible that spontaneous spatial novelty preference may be impaired if rats underwent multiple exposures.

Aim 2: To better understand the conditions that determine the magnitude of effect or expression of the phenotype of anaesthetic-induced cognitive impairment

Three conditions were investigated, (i) the relative effects of single and multiple anaesthetic exposures, (ii) the relative effects of different anaesthetic drug regimens and (iii) the effects of anaesthetic exposure at different developmental time points. Anaesthetic exposure during adulthood, to multiple episodes of anaesthesia, was found to be more detrimental to win-shift radial arm maze performance than a single episode (Chapter 3). This was despite a one-week 'wash out' period between successive anaesthetics, and so is not due to a straightforward cumulation of anaesthetic drugs. Despite this increase in the magnitude of effect on win-shift spatial memory, multiple exposures were not associated with impairments in attentional processing, regardless of whether a volatile based anaesthetic regimen (isoflurane plus nitrous oxide) or an injectable anaesthetic regimen (propofol plus fentanyl) was used (Chapter 4). Indeed, despite a mild impairment in 5CSRTT performance, induced by a single episode of a volatile-based anaesthetic regimen, no impairment was detected in rats that had received multiple exposures.

This phenotypic pattern of impairment (impairment in spatial memory but not in particular aspects of attention, the magnitude or expression of which is dependent on the number of anaesthetic exposures) was mirrored in experiments investigating the

effects of anaesthetic exposure during development. Multiple, but not single, exposures to anaesthesia during development, produced win-shift spatial memory deficits, when testing subsequently occurred in adulthood (Chapter 5). In contrast, neonatally exposed rats did not demonstrate impairments in attention (as assessed with the sustained attention and the intra-dimensional / extra-dimensional shift tasks), regardless of the number of exposures.

Notably however, impairment in win-shift radial arm maze performance, following a single anaesthetic exposure, was only seen when rats were exposed during adulthood (Chapter 3) and not development (Chapter 5); suggesting perhaps that developmental exposure is less detrimental than exposure during adulthood - at least for the performance of certain tasks. However, it must be noted that experiments in Chapter 3 investigated the effects of isoflurane plus nitrous oxide, whereas those in Chapter 5 investigated the effects of isoflurane in the absence of nitrous oxide. Nitrous oxide has been associated with anaesthetic-induced neurotoxicity and cognitive impairment when administered during development (Jevtovic-Todorovic et al. 2003b) and during adulthood (Culley et al., 2004b), therefore the disparity in the magnitude of post-anaesthetic cognitive impairment seen here in Chapters 3 and 5 are perhaps more likely a result of this variation in the anaesthetic regimen used.

Aim 3: to better understand the neuronal mechanisms underpinning anaesthetic-induced cognitive impairment

Despite the demonstration, in Chapter 3, of an anaesthesia-induced deficit in a task that is dependent on function of the hippocampus (the win-shift radial arm maze task); anaesthetic exposure, during adulthood, to 2 hours of isoflurane plus nitrous oxide

anaesthesia did not alter the total dendritic length or arborisation of CA1 hippocampal cells. Anaesthetic exposure also did not alter the density of spines on apical dendrites, however it was associated with a numerical increase in spine density, on basal dendrites, that did not quite reach statistical significance. These results do not provide compelling evidence for changes to dendritic ultrastructure, as a neuronal mechanism for anaesthetic-induced cognitive impairment, following exposure in adulthood.

Collective consideration of the results

Does anaesthesia cause enduring cognitive impairment?

The results of this program of work demonstrate that 2 hours of anaesthesia, in the absence of surgery, is sufficient to induce a deficit in short term spatial memory, and that the magnitude of that effect is dependent on the number of exposures. These results are in line with existing literature (Culley et al., 2004b, Crosby et al., 2005) and support the view that anaesthesia can indeed cause enduring cognitive impairment.

The impairment demonstrated occurred relatively soon after the administration of anaesthesia (between 48 hours and 9 days post-anaesthetic exposure) - long enough for the anaesthetic agents to have been cleared from the body, but perhaps not long enough for task performance *not* to be affected by disruption of sleep. Given that sleep disturbance is associated with subsequent memory impairment, in rodents (Binder et al., 2012, Inostroza et al., 2013) and humans (Ratcliff and Van Dongen, 2009, Nebes et al., 2009), it could be viewed that anaesthetic-induced cognitive impairments could, at least partially, be related to sleep disturbance. Anaesthetic-induced alterations have been associated with a phase delay in the circadian clock (Cheeseman et al., 2012, Challet et al., 2007). Evidence from a recent study carried out in honey bees suggests that anaesthesia is only associated with sleep disturbance if administered during the active phase of a 24 hour period (Cheeseman et al., 2012). These data suggest that rats anaesthetised in the light / inactive phase (as in Chapter 3 and (Culley et al., 2004b)) might *not* be expected to demonstrate cognitive impairment. On this basis either sleep disturbance happens when anaesthesia occurs during the light phase also, or it is not

a factor. In addition, consideration of the attention results supports that sleep disturbance is not a factor in anaesthetic-induced cognitive impairment. Sleep deprivation causes impairments in the 5CSRTT (Cordova et al., 2006) and in the IDED task (McCoy et al., 2007). Although impairment in 5CSRTT was seen in rats exposed to anaesthesia in adulthood (Chapter 4), the magnitude of this impairment did not increase with more exposures (and more potential disruption to sleep). Further to this no impairment of IDED performance was seen in rats exposed to anaesthesia during development (Chapter 6), again supporting that sleep disturbance is not a factor in the anaesthetic-induced cognitive impairments demonstrated in other tasks.

Specific investigation of sleep patterns in models of anaesthetic-induced cognitive impairment would help to clarify the extent to which such a physiological disturbance, versus toxicity *per se,* may contribute to the phenotype.

The psychological basis of anaesthetic-induced impairment in win-shift radial arm maze performance?

It was demonstrated that 2 hours of exposure to isoflurane and nitrous oxide anaesthesia was sufficient to impair performance of a win-shift radial arm maze task in young, healthy, adult rats. Win-shift spatial memory (assessed on the radial arm maze) is a form of short-term memory. The precise psychological processes involved in successful completion of the task are still debated (Sanderson and Bannerman, 2012, Sanderson et al., 2010), but here it is shown that the same anaesthetic regimen did not impair spatial novelty preference or aspects of attention (both candidates for processes involved in completion of the task).

A single exposure of the same anaesthetic regimen *was* associated with an increase in the number of omitted trials on the 5CSRTT, suggesting that attentional function could be adversely affected by anaesthetic exposure. The magnitude of the impairment did not increase with successive exposures though (unlike the impairment of radial maze performance), and so the extent to which a gross derangement of attention could drive the radial arm maze impairments is unclear. It is of note however that rats tested on the 5CSRTT underwent training before the first anaesthetic exposure, and behavioural testing between successive anaesthetic exposures, which likely induced plasticity (unassociated with anaesthetic exposure) and may have partially offset the effect of anaesthesia.

Why is it important to understand the psychological basis of anaesthetic-induced cognitive impairment?

In order to establish the neurobiological mechanisms that underlie anaesthetic-induced cognitive impairment, it is necessary to understand the psychological basis of the condition. The neurobiological mechanisms underlying an impairment in spatial memory, novelty preference or attention for instance, may be very different. Characterisation of the psychological processes impaired following anaesthesia, would therefore enable the design of experiments to better target the anatomical or molecular correlates.

Such an approach is also important to ensure that the same psychological processes are being studied in humans and animals, ensuring that results within the field are fully translatable. If the aim of animal studies is to model the human condition, then we need to ensure that the same psychological processes are being studied in both cases.

Precisely how the win-shift radial arm maze task relates to tasks used to investigate human POCD, is unclear. Certainly deficits in visuo-spatial memory have been detected in human patients, but more commonly with computerised tasks such as the spatial span task. This task is a spatial working memory test in which patients sit before a screen containing a number of illuminated squares (Bor et al., 2006). The squares briefly change colour in a variable sequence and participants are required to touch the squares in the same sequence that they change colour. The spatial span task requires subjects to 'hold' information in their short-term memory, as does the RAM task. However, the psychological processes involved may be very different to that of RAM task, not least because it does not contain a navigation component. Virtual reality spatial navigation tasks have been used to detect impairments in patients suffering from traumatic brain injury (Skelton et al., 2006) but they have not yet been used within the field of POCD.

The spatial span task has some similarity to the 5CSRTT used here to investigate post-anaesthetic attention in adult rats, although the spatial component is less complex. This is because the spatial span task requires subjects to hold multiple locations in mind, in a specific sequence, before making a response; where-as the 5CSRTT requires that only a single location is identified before responding. Reaction time tasks are commonly included in neuropsychological test batteries, including that established by the International Study of Post-operative Cognitive Dysfunction (ISPOCD: (Moller et al., 1998, Price et al., 2008, Johnson et al., 2002, Monk et al., 2008). A number of variations exist but the basic premise is that subjects signal a response to one or more presented visual stimuli, and the time taken to respond is recorded. The scoring of these tasks varies between studies (with some studies scoring the number of errors, some scoring the time taken to make a choice, and some using

a composite score). Here the impairment in 5CSRTT performance, seen in rats that received a single anaesthetic exposure during adulthood, was characterised by an increase in omissions, rather than either errors or time taken to make a choice. This measure could potentially be affected by factors such as motivation to make a choice. Although rats here did not differ in the time taken to either make a choice or collect food reward, suggesting that motivation was not affected by anaesthesia. An increase in omissions could also potentially be related the error rate and time to make a choice, by reflecting a 'trade-off' with these performance measures.

The intra dimensional / extra dimensional shift task (IDED) used here to investigate aspects of post-anaesthetic attention in neonatal rats, was similarly chosen because a human homolog (described in Chapter 6) has been used to investigate the effects of anaesthetic exposure. Healthy human subjects given sub-anaesthetic doses of ketamine demonstrate increases in the percentage of perseverative and non-perseverative errors and the number of trials to completion of the Wisconsin Card

Sorting Task (Krystal et al., 2000, Krystal et al., 1994). The results of this study are not directly comparable to the data presented here, because they examined attentional performance whilst the drug was still being administered. The Wisconsin Card Sorting Task task is thought to assess cognitive flexibility, a domain that has been investigated in patients with POCD with the use of another task - the concept shifting task. In the concept shifting task subjects are presented with a mixture of letters and numbers and are required to rapidly shift between them, as they order them. Impairments in post-operative cognitive function, within the first week of surgery, have been detected using this task in older patients (Price et al., 2008).

Attention is a heterogeneous construct, and so the relative lack of impairment in the specific tasks used within this thesis, does not rule out an effect of anaesthesia on some other aspect of attention. However, it is surprising given that impairments, in similar tasks, have been detected in human patients following anaesthetic exposure. It could be that such impairments in attention are related more to the challenge of surgery and hospital stay.

Limitations to conclusions about the phenotype

The phenotype of anaesthetic-induced cognitive impairment in adult rats has been further characterised through the elimination of particular explanations. However, altered performance of the win-shift radial arm maze task could still potentially involve effects on a range of other sensory and cognitive and processes (such as behavioural inhibition, arousal, anxiety, motivation or sensorimotor function) not tested here. Impairment in one of these processes could account for impairment of task performance following anaesthesia. Of note, the same processes might be expected to equally affect performance of other tasks. For instance, anaesthetic-induced anxiety would presumably similarly affect exploration of the spontaneous spatial novelty preference (SSNP), Y maze, task and the RAM task. However, an impairment in SSNP was not found. Use of a standardised battery of tests, that comprehensively cover multiple sensory and cognitive processes, could better facilitate comparison both within and between studies.

Factors affecting the expression of anaesthetic-induced cognitive impairment

Life stage

The suggestion here that exposure to anaesthesia during development is less detrimental than exposure during adulthood, is at odds with existing literature (Culley et al., 2007, Jevtovic-Todorovic et al., 2013). The effects of manipulations during development often depend on the precise timing of the manipulation. The developmental time points chosen here, for anaesthetic exposure (P7-P13), have been previously associated with anaesthetic-induced cognitive impairment and/or toxicity and plasticity (Jevtovic-Todorovic et al., 2013). It therefore seems unlikely that the relative lack of effects seen during development are a function of this feature of experimental design.

Direct comparison of the results with those from rats exposed during adulthood may, however, be confounded by the choice of agent. Neonatal rats were exposed to isoflurane alone, and yet adults were exposed to isoflurane plus nitrous oxide. The neurotoxic effects of nitrous oxide have been established (Jevtovic-Todorovic et al., 1998, Jevtovic-Todorovic et al., 2003a, Jevtovic-Todorovic et al., 2001) and its use has been associated with persistent learning deficits (Jevtovic-Todorovic et al., 2003b). It is possible that the absence of nitrous oxide from the neonatal anaesthetic regimen accounts for the anomaly observed here (see later for a discussion about the relative merits of single agent and multiple agent anaesthetic protocols).

Of course one important difference between the experiments conducted following anaesthetic exposure during adulthood and during development, was the time between anaesthetic exposure and behavioural testing. Adult exposed rats were tested from 48 hours after anaesthetic exposure, where-as those exposed during development were

tested 3 months after exposure. It is therefore possible that although brief exposures to anaesthesia during adulthood can be sufficient to produce cognitive deficits, they deficits may be limited in the extent to which they are enduring, and may not have been detected at 3 months after exposure. Indeed, young adult patients demonstrate POCD at one week but not 3 months post-surgery (Johnson et al., 2002).

Animal husbandry

The variation in animal husbandry lighting protocols (testing during the light/inactive phase in Chapters 3 and 6, and Experiment 1 Chapter 4) and testing during the dark/active phase for other chapters) was unfortunate, and was a function of facility limitations. There is conflicting literature on the role that this variable has on behavioural testing results, with some studies finding that rodent testing during the light phase are impaired compared to those tested during the dark phase (Takahashi et al., 2013, Gritton et al., 2009, Gritton et al., 2012) and some finding the opposite to be the case (Binder et al., 2012, Chaudhury and Colwell, 2002, Rawashdeh et al., 2014). Such mixed results serve to support the notion that optimal experimental design would involve standardisation of this variable.

Environmental enrichment

The lack of impairment in win-shift spatial radial maze performance following anaesthetic exposure during development, as compared to adulthood, is surprising (see 'Life stage' section above). Importantly, spatial memory was tested at time point in adulthood (3 months after exposure) that is later than generally used when studying anaesthetic-induced cognitive impairment. If the effects of anaesthesia are transient this could account for the relative lack of effects detected in radial maze performance.

However, one other consideration is the effect of environmental enrichment on expression of the phenotype. Throughout the experiments of this thesis, the type of environmental enrichment was standardised (to bedding, nesting material and a tube that subjects could escape to), in an attempt to reduce between study variation. However, when comparing the results from rats exposed during development here, with results from other laboratories, differences in the level of enrichment may be more of a consideration.

Guidelines for the reporting of animal experiments (published by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (McGrath et al., 2010) recommend that details of environmental enrichment are included in all publications. Most publications, within the field of developmental anaesthetic neurotoxicity, do not yet contain detailed information on environmental enrichment, however, discussion with co-members of the 'Preclinical Pediatric Working Group' (an initiative from the Federal Drug Agency and the International Anesthesia Research Society), reveals that standard housing conditions for many studies are relatively barren (containing only bedding material). Given that increased enrichment is associated with the amelioration of behavioural deficits in a variety of animal models (for review see (Burrows and Hannan, 2013)), and that the effects of enrichment might be more pronounced during development (Girbovan and Plamondon, 2013) this factor could provide a confound, for study comparison. Indeed, rats exposed to sevoflurane during development, and subsequently housed in an environment containing increased enrichment, demonstrated no impairment in short term spatial memory compared to anaesthetic exposed rats that were housed in standard conditions (Shih et al., 2012).

Stress

Another animal care factor that could confound results is stress, for instance in response to husbandry or handling practices, or stages of the experiment such as maternal separation (in the case of studies using neonatal animals). Stress can affect animal models in a variety of ways, including the induction of alterations to the function of the hypothalamic-pituitary-adrenal axis and molecular modifications that alter gene expression (Harris, 2015, Gudsnuk and Champagne, 2012). For the experiments presented here, housing and husbandry methods involved acclimating animals to handling as well as changes to standard husbandry practice (such as the experimenter being the person to change cages during testing periods, rather than relatively unknown animal care staff). These methods were implemented with the aim of reducing stress for the animals and are in line with the principle of refinement. No specific hypothesis about their effect (or not) is made. However, it is of note that such methods are not invariably applied within the field (personal communication with co-members of (Preclinical-Pediatric-Anesthesia-Working-Group, 2015)) and this variability could complicate direct cross study comparison.

Sex of the subjects

Both male and female neonatal rats were tested on the radial arm maze. Previously anaesthetised female rats made less choices before the first error occurred than male rats, suggesting that they were relatively impaired. In a recent study, assessing spatial reference memory in the Morris Water Maze, following anaesthetic exposure during development, female rats were slower to acquire the same level of performance as male rats (Boscolo et al., 2013b), supporting the findings here that cognitive impairment may be sex-specific. However, as sex differences were not the primary focus here,

the study may not have been powered adequately to enable that aspect of the result to be conclusive. This factor could be more comprehensively studied in future experiments.

Neuronal mechanisms underpinning anaesthetic-induced cognitive impairment

The lack of anaesthetic effect on hippocampal dendritic ultrastructure could be considered surprising, given that one of the most robust findings following anaesthetic exposure is impairment of hippocampal dependent memory tasks, and that changes have been demonstrated in other brain areas (Briner et al., 2011, De Roo et al., 2009). Anaesthetic-induced biochemical changes in the hippocampi of young, adult, rats have been demonstrated. For example, a study investigating the underlying neuronal mechanisms in a model of anaesthetic-induced impairment in fear conditioning, found decreased neuronal density in the CA1 area of the hippocampus (the same anatomical area as studied here). They also found that levels of an inflammatory cytokine (interleukin 1-beta) were increased throughout the hippocampus, following anaesthetic exposure (Lin and Zuo, 2011). It is not the case, therefore, that the hippocampus is unaffected by anaesthetic exposure. Alterations in the density of dendritic spines may not therefore be as sensitive to anaesthesia when exposure occurs during adulthood, or statistical power for this study may simply have been insufficient to detect any but the most dramatic effect.

Exposure to 2 hours of sevoflurane anaesthesia (an agent that is similar to isoflurane) during development is associated with synaptic loss in adulthood (as assessed with electron microscopy) within the rat hippocampus (Amrock et al., 2015). Multiple

exposures (but not one long exposure of equivalent total duration) of the same anaesthetic regimen were associated with a greater degree of synaptic loss. This increase in the magnitude of effect parallels that of the anaesthetic-induced impairment in win-shift radial arm maze performance, demonstrated here (Chapter 3). Future experiments investigating the mechanisms underlying this impairment could therefore include this method of analysis, along with further study of dendritic spine density.

Experimental design considerations

The anaesthetic regimen chosen

All experiments investigated the effect of a set dose of anaesthesia rather than determining a behavioural endpoint associated with depth of anaesthesia. Individual variation in absorption, distribution, metabolism and elimination of the drugs may have increased variance within experiments. Future work could standardise the depth of anaesthesia, for instance by using a mechanical threshold device (Raper, under review), in order to ensure that all subjects are anaesthetised to the same behavioural endpoint. It could be considered that this has more relevance to the resulting effects than the dose of agent administered.

The anaesthetic regimens used to investigate anaesthetic-induced cognitive impairment in adults involved exposure to combinations of drugs, as opposed to a single agent used for investigation in the neonatal studies. Combination regimens more closely model the clinical situation, where multimodal approaches are used in order to limit the dose requirements (and therefore side effects) of each individual agent. And this approach was chosen as the starting point (in adult rats) for this program of work based on pre- existing literature within the field.

Single agent regimens were chosen for examination of the effects of anaesthesia during development in order to facilitate building on existing literature within that field, which now tends to focus on single agent effects in order to better facilitate mechanistic effects. Although with regard to the relative effects of anaesthetic exposure during development, demonstrated here and those within pre-existing literature, it is of note

that robust impairments following exposure to a combination of anaesthetic agents is well established (for example (Jevtovic-Todorovic et al., 2003b)), however the effects of single agent exposure are not invariable.

This difference in approach, between adult and neonatal studies could be viewed as a limitation of the work presented here, by limiting the extent to which direct comparisons can be made. However, the use of single agent protocols, and indeed the change from isoflurane (Chapter 5) to sevoflurane (Chapter 6) (which became of greater interest as the field progressed within the life time of this program of work), facilitates comparison with a rapidly expanding wider literature on the effects of anaesthetic exposure during development. Of note, investigation of the effects of sevoflurane on spatial memory was undertaken, along with examination of the relative effects of multiple short exposures and a single exposure of the same total duration (data not shown). However, unfortunately due to hospitalisation of the experimenter (and author here), behavioural testing could not be completed. Investigation of the relative effects of different duration exposures could provide important information for the field of behavioural neuroscience, for instance, where neonatal animals undergo imaging procedures throughout development.

Physiological stability

Blood gas results from neonatal rats demonstrated the occurrence of mild acidaemia. This is in line with pre-existing literature, where anaesthesia of neonatal rodents is sometime associated with marked physiological deviations (Vutskits and Patel, 2014). Here it was important to model typical laboratory animal conditions, and so

intubation and mechanical ventilation were considered inappropriate. It has been argued that for results to be translatable to human medicine, such support of respiratory function is needed (Vutskits and Patel, 2014). These methods could be considered for future experiments. Although given how technically challenging they are in small patients and the fact that in some species intubation is associated with a higher risk of anaesthetic-related mortality (Brodbelt et al., 2007), it may be a long time before they are standard practice within the rodent field.

Sample size

It is important to note that the sample sizes chosen for the experiments contained within this thesis were relatively small. Coupled with the unforeseen sample size losses encountered in some of the chapters (especially Chapter 7), it must be considered that the statistical power for these experiments may be too low for the conclusions to be robust. The statistical power (or sensitivity) of a test is the probability that the test will correctly reject the null hypothesis, and depends on the construct of the test (significance level chosen), the magnitude of the effect size within the population, as well as the sample size being tested (Button et al., 2013). All efforts were made to reduce measurement error and varience when designing experiments, in order that lower sample sizes could be used and in line with the principle of reduction. However, small sample sizes lead to a decreased liklihood that effects present within the population will be detected (type II errors), and to an increased liklihood that false effects will be detected (type I errors). The conclusions within this thesis must therefore be interpreted in the context of this limitation.

Future directions

The follow up experiments suggested thus far would facilitate further investigation of the role of anaesthesia as a possible confound for behavioural neuroscience experiments in rodents. In addition, an area of future interest is the phenotypic characterisation of anaesthetic-induced cognitive impairment in non-human primates, in order to provide a model of POCD with greater translatability to humans. Whether general anaesthesia is neurotoxic to infants and children, and may result in long-term neurocognitive impairments, is one of the most critical questions in paediatric anaesthetic practice today. How well the cognitive deficits demonstrated in rodents translate to human clinical practice is questioned (Bhutta et al., 2007, Loepke and Soriano, 2008). Partly this view relates to the uncertainty with which developmental stages can be compared between rodent and human species. The neurodevelopment of non-human primates is more similar to that of humans (Clancy et al., 2007a, Brambrink et al., 2010) and so may provide a more relevant model.

Both anaesthetic-induced neurotoxicity (Slikker et al., 2007, Zou et al., 2011), and cognitive impairment (Paule et al., 2011) have been demonstrated in non-human primate studies, adding weight to the possibility that anaesthetic exposure could adversely affect the development of human infants. Infant monkeys exposed to ketamine within the first week of life, demonstrate multiple impairments in appetitively motivated tasks, such as the incremental repeated acquisition task (that requires subjects to learn a different series of lever presses during each test session). Monkeys were tested from 7 months old for 136 weeks in total, suggesting that impairment was enduring (Paule et al., 2011). However, the group that was previously anaesthetised also demonstrated impairment in motivation (as assessed with a

progressive ratio task) and so it is not possible to rule out the possibility that their performance was confounded by lower motivation. Further investigation to build on this initial finding, is therefore needed.

A non-human primate model of cognitive development after early exposure to anaesthesia could bridge the gap between results obtained in rodents and human clinical practice, as some psychological processes are difficult to study in rodents. To that end, the data from Chapter 5 here, was presented in support of a successful grant application to the National Institute of Health, USA (grant code 1R01HD068388-01). That program of work investigates the cognitive development of macaque monkeys exposed to three episodes of sevoflurane anaesthesia. Initial results (under review) demonstrate that anaesthetised monkeys exhibited increased anxiety and hostility reactions, five months after exposure, supporting the view that anaesthetic exposure may induce effects relevant to human clinical practice. Future behavioural testing of these monkeys will involve more complex behavioural tasks, including those that test spatial memory and other aspects of cognitive and socio-emotional function.

Final thoughts

It is not the case that anaesthesia can be avoided during the course of behavioural neuroscience experiments that contain a potentially painful or distressing component. Indeed, aside from the obvious welfare implications of such a suggestion, both pain and stress also can induce plasticity (e.g., (Kuner, 2010)) and physiological changes (e.g., (Goldkuhl et al., 2010)) that could change animal models and confound scientific output. What is important therefore is that anaesthesia is used where needed, and study design accounts for the potential effects of anaesthesia by including anaesthesia only control groups where necessary.

APPENDIX 1: OVERVIEW OF THE LEGAL FRAMEWORK GOVERNING ANIMAL RESEARCH IN THE UNITED KINGDOM

Within the UK, personnel conducting scientific procedures on laboratory animals must be granted a Procedure Individual Licence (also called a Personal Licence), under authority of the Animals (Scientific) Procedures Act 1986; as amended 2012 (ASPA, 1986). The Procedure Individual Licence is subject to a set of standard conditions that place certain legal obligations upon the holder. For instance, Standard Condition 9 requires researchers to use anaesthesia, as the default position, when carrying out experimental procedures that might cause pain. Further to that, Standard Condition 4 obliges researchers to use anaesthesia for procedures that might cause distress e.g., imaging procedures or other such minor procedures that would not necessarily be expected to induce pain, and might be carried out without anaesthesia within a clinical setting.

As well as the use of anaesthesia by default, researchers are required to continually refine (improve) anaesthetic techniques and protocols, an obligation referred to in Standard Condition 1, and one that requires researchers to keep abreast of best practice. Other countries may not have such a detailed legislative framework but these ethically driven obligations are still represented within legislation and guidelines (e.g., (National-Research-Council, 2011, Peoples-Republic-of-China, in prep, Directive, 2010/63/EU).

APPENDIX 2: DESCRIPTION OF THE STUDY THAT FORMED THE IMPETUS FOR THIS THESIS WORK

Whilst working with researchers to refine the anaesthesia for a behavioural neuroscience model, a question was raised about the possible use of propofol (a phenol anaesthetic agent) as an adjunct, to volatile anaesthesia for excitotoxic brain lesion surgery in macaques. There were a number of potential clinical benefits associated with the proposed refinement but a lack of data on any effects the refinement may, or may not, have on the experimental output (i.e., effectiveness of the excitotoxic agent which could impact on the post-surgical behavioural testing results).

At the time that this refinement was being considered there was an observation, in another institution, that a neurotoxic lesion was ineffective in a single marmoset anaesthetised with propofol, instead of alphaxalone/alphadalone (personal communication Taylor 2006). The anecdote raised the possibility that propofol anaesthesia might interfere with the induction of an experimental brain lesion. In order to investigate this further I collaborated on a project to determine whether propofol prevents the intended behavioural impairment resulting from a bilateral neurotoxic dorsal hippocampal brain lesion in rats (a well described model of spatial memory impairment, e.g., (Moser et al., 1995). Rats were given precise surgical brain lesions under either propofol or isoflurane anaesthesia (ensuring physiological stability throughout). Post-operative spatial memory was assessed with a 3/6 reference/working memory task on a radial arm maze, where three of the 6 arms of

the maze were baited with food reward (as in (Schmitt et al., 2003)). This task is particularly sensitive to dorsal hippocampal damage (Pothuizen et al., 2004). In order to collect the three food rewards (3 out of 6 arms were baited) efficiently rats must make use of extra maze cues, both within and across trials, in order to avoid previously visited arms and learn which 3 arms are baited. These two separate psychological processes are commonly termed working and reference memory (respectively) and are dependent on different neurobiological mechanisms. Rats performed one trial per day for 21 days, beginning 15 days after surgery. Results showed that propofol did *not* prevent the intended behavioural impairment resulting from the brain lesion or the effectiveness of the lesion itself in destroying hippocampal neurons. In other words the results did not support our hypothesis formulated from the anecdotal report. However, although the lesion impaired radial maze performance regardless of whether propofol or isoflurane was used during placement of the lesion, the pattern of impairment over the training period differed depending on the anaesthetic used. Impairment emerged earlier in training in the propofol-lesion group than in the isoflurane-lesion group (Fig 1A), despite a similar volume of lesion in both groups. Where the volume was determined by plotting the lesion area of serial sections, on to standard rats brain sections (Paxinos and Watson, 2005). There were no group differences in mean arterial blood pressure during the surgical procedure, or in venous blood gases collected at the end of surgery, so intra-operative hypoxia doesn't account for this effect. (see (Baxter et al., 2008) for full discussion). It is not clear whether propofol anaesthesia exacerbated the effect of the hippocampal lesion, or isoflurane retarded the emergence of behavioural impairment following the lesion. In either case, the results imply that the administration of general anaesthetics to normal, young adult animals may have an impact on central

nervous system function, long after the acute pharmacological effects of anaesthesia have dissipated.

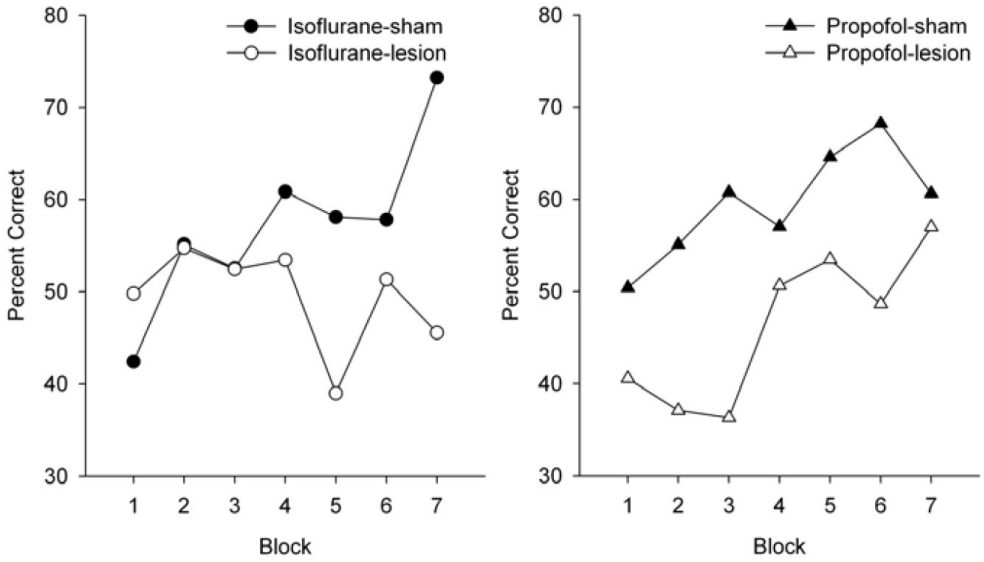

Figure 1A (Baxter et al., 2008). Rats with neurotoxic dorsal hippocampal lesions placed under either isoflurane or propofol anaesthesia began testing on a 3/6 working/reference memory task 14 days after surgery. The 'percent correct' measure represents the percent of arm choices in each session that resulted in reward delivery. Each session was terminated when all 3 rewards were obtained or after 10 minutes. Data are grouped into 3-day blocks. Rats that received their lesion under propofol appear more severely impaired at the beginning of training. The interaction of anaesthesia, lesion and block is significant ($F[6,132] = 2.42$, $p = 0.03$). There are no significant differences in performance of the sham groups. N=7 in each lesion group and N=6 in each sham group.

The unexpected interaction observed between anaesthesia and the surgical procedure may have important implications for the design of *in vivo* behavioural neuroscience experiments. In particular, variations in anaesthetic protocols between laboratories may contribute more to inter-study differences than has been appreciated previously. Control animals in the described experiment received sham surgery (matched with lesion groups for duration) under either propofol or isoflurane anaesthesia and did not demonstrate an anaesthetic agent-dependent difference in post-operative spatial memory performance. In other words there was no main effect of anaesthetic agent *per*

se on post-operative spatial memory performance; rather the effect was on the expression of the lesion deficit. However, the experiment was not designed to investigate the effects of anaesthesia alone and did not include anaesthetised or naive control groups that did not receive surgery. It was therefore not possible to determine the contribution (if any) that the anaesthetic agents would have made to the post-operative behavioural performance in the absence of surgery.

The results of the above experiment raised questions around the role of anaesthesia as a potential confound for behavioural neuroscience experiments. There are a number of ways that these questions could be followed up. This thesis follows one line of investigation by examining the effects of anaesthesia, *in the absence of surgery*, on post- anaesthetic cognitive function.

APPENDIX 3: THE INCEPTION OF MODERN DAY ANAESTHETIC PRACTICE

Animals have been used to advance scientific knowledge from as far back as 6th BC (Franco, 2013). The scene famously (or perhaps infamously) painted by Emile Edouard Mouchy in 1832 (Fig 1B) appears to depict the performance of an invasive surgical procedure on a conscious dog - a commonplace necessity in the days before modern anaesthetic methods. There remains no doubt that much was learned from these historical experiments but in modern times many would consider such a cost (in terms of animal suffering) to be unacceptably high, even barbaric.

Figure 1B: Painting from the Wellcome Library. A Physiological Demonstration with the Vivisection of a Dog by Emile Edouard Mouchy, painted in 1832.

In 1846 (just a few years after this scene was painted) the dentist William Morton reached notoriety for publicly demonstrating the use of inhaled ether to facilitate surgery, a monumental event that sparked the creation of anaesthesia as a discipline. William Morton's claim of being the discoverer of anaesthesia is contentious however, with at least three others staking claims at around the same time (Wolfe, 2000). What is less well known, is that a demonstration of intravenous anaesthesia (a mixture of opium and alcohol) from which a full recovery was made, was performed on a dog in the 1600s by Robert Boyle, a physiologist (Hunter, Unpublished Writings, 1645–c1670). Correspondence reports from that time, exerts of which are contained in (Dorrington and Poole, 2013), illustrate just how very close the researchers were to recognising the importance of their experimental results, for laboratory animal, and human, welfare. But unfortunately, it was to be almost 200 years before modern day anaesthetic practice was born.

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