General Anaesthetic Modulation of Memory-Related Gene Expression Using an *In vitro* Brain Slice Model

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Abstract

General anaesthetics cause widespread neurochemical and physiological changes in the brain. However, the precise mechanism of amnesic action is largely unknown. Gene expression changes in the hippocampus have been a focal point for investigation in this area, while effects on the cerebral cortex have been largely underreported even though the cerebral cortex has been shown to play a large role in memory consolidation and storage. Amnesia is likely due to the change in expression of memory-related genes within the neocortex or hippocampus of the mammalian brain.

The first aim of this research was to investigate the *in vitro* cortical gene expression pattern of two memory-related genes; *a*ctivity-regulated cytoskeleton-associated protein (*Arc*) and brain-derived neurotrophic factor (*Bdnf*), after a *t=4 hour* exposure to propofol- or sevoflurane- induced anaesthesia using an adult mouse brain slice model and real-time quantitative PCR. Five animals were used for each anaesthetic and a *t=0 hour* control*, t=4 hour* control and *t=4 hour* treated 400 µm slice were taken from each animal. Seizure-like activity was recorded from the brain slices to ensure viability of the tissue before carrying out the anaesthetic exposure. RNA was extracted, DNAse-treated, and then converted to cDNA. Quantitative PCR was then carried out to analyse *Bdnf* and *Arc* expression differences between the *t=4 hour* control and *t=4 hour* treated samples using *Gapdh*, *β2m*, *Actb* and *HRPT1* as reference genes. The second research aim was to determine the Bdnf protein expression level and localisation after a *t*=4 hour exposure to propofol using western blot and immunohistochemistry methodologies.

Our research demonstrated that *Arc* was significantly down-regulated after exposure to sevoflurane for *t=4 hours* ($p<0.05$)*. Arc* was also shown to be up-regulated after a *t=4 hour* exposure to propofol while *Bdnf* showed a downregulation to sevoflurane but an upregulation to propofol, however this

data was not statistically significant. Western blot data showed that the rabbit polyclonal Bdnf antibody was binding to an off-target epitope at 55 kDa with mouse brain, heart, lung, liver, spleen and kidney whole tissue lysate. A newly sourced commercial Bdnf antibody was validated and western blot data showed recognition of the Bdnf epitope at the correct predicted size of 28 kDa in the mouse brain, heart and kidney. Immunohistochemistry of frozen mouse brain sections failed to produce a positive signal for two different Bdnf antibodies due to encountering technical issues. Hematoxylin and Eosin staining showed the tissue was still intact after the sectioning procedure. These issues could be resolved in the future by improving the tissue fixation length and optimising the antigen retrieval step.

Eleven recommendations have been made to provide further insight into the gene expression levels of memory-related genes in the mouse brain. This includes varying the induction and maintenance of the two anaesthetic drugs, role of epigenetic modification, evaluating the mental state of the mice (depression), investigating Bdnf knockout, differential expression between areas of the brain and brain electrical activity. In addition, the gene expression pattern after a period of anaesthetic exposure should also be analysed. This may help elucidate the causes of postoperative cognitive dysfunction. Whole transcriptome analysis using RNA-Seq could be used to determine the expression levels of other known memory-related genes after anaesthetic exposure.

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List of Abbreviations

5-HT	Serotonin receptor
α	Alpha
Δ	Change in
ΔΔCT	Delta delta C_T (equation)
µL	Microlitre
µM	Micromole
µm	Micrometre
Actb	Beta-actin
nACSF	Normal artificial cerebral spinal fluid
Akt	Protein kinase B protein
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid protein
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor protein
APS	Ammonium persulphate
Arc	Activity-regulated cytoskeleton-associated protein gene
Arc	Activity-regulated cytoskeleton-associated protein
AS	Antisense
Avp	Arginine vasopression gene
β2m	Beta-2 microglobulin
Bdnf	Brain-derived neurotrophic factor gene
BDNF	Brain-derived neurotrophic factor protein
BGI	Beijing Genomics Institute
bp	Base pair
BSA	Bovine serum albumin protein
CA	*Cornu ammonis*
Ca^{2+}	Calcium ions
cDNA	Complementary DNA
Chrna3	Acetylcholine receptor alpha-3 gene

Cl^-	Chloride ions
CNS	Central nervous system
CREB	Cyclic AMP response element-binding protein
C_T	Cycle threshold
DAPI	4', 6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DHPG	Dihydroxyphenylglycine
DNA	Dioxyribose nucleic acid
dsDNA	Double stranded DNA
E	Efficiency
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
EJC	Exon junction complexes
ERK	Extracellular signal-regulated kinase protein
EtBr	Ethidium bromide
FCS	Fear Conditioning System
g	Gram
GABA	Gamma-aminobutyric acid
$GABA_A$	Gamma-aminobutyric acid alpha receptor protein
$GABA_{A4}$	Gamma-aminobutyric acid alpha-4 receptor protein
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase gene
gDNA	Genomic DNA
GOI	Gene of interest
HFS	High-frequency stimulation
hnRNP	Heterogeneous nuclear ribonucleoprotein
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase gene
HRP	Horse radish peroxidase protein
Hz	Hertz
IEG	Intermediate early gene
IGF-1	Insulin-like growth factor-1 gene
IV	Intravenous

kB	Kilobases
kBp	Kilobase pairs
KO	Knockout
LFP	Local field potential
LTD	Long-term depression
LTP	Long-term potentiation
MAC	Minimum alveolar concentration
MAP2	Microtubule-associated protein 2
MAPK	Extracellular signal-regulated kinase protein
Mef2	Myocyte enhancer factor 2 protein
mg	Milligram
Mg	Magnesium
mL	Millilitre
mM	Millimole
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
mQ$H_2$0	Milli-Q $H_2$0
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NGF	Nerve growth factor
nM	Nanomole
NMD	Nonsense-mediated decay
NMDA	N-methyl-D-aspartate protein
NMDAR	N-methyl-D-aspartate receptor protein
NT-3	Neurotrophin-3 protein
NT-4	Neurotrophin-4 protein
NTC	No template control
OCT	Optimum cutting temperature, frozen section compound
ORF	Open reading frame
OT	Oxytocin neurophysin gene
$p75^{NTR}$	p75 neutrophin receptor protein

PEST	Proline, glutamic acid, serine, threonine sequence
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3-K	Phospoinositide 3-kinase protein
POCD	Post-operative cognitive dysfunction
PSD	Postsynaptic density
PTSD	Posttraumatic stress disorder
PVDF	Polyvinylidene difluoride
qPCR	Real-time quantitative polymerase chain reaction
QC	Quality control
QT	Q-wave T-wave
R	Relative expression ratio
RIN	RNA integrity number
Rf	Reference gene
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNases	Ribonuclease
RT	Room temperature
-RT	No reverse transcription control
SARE	Synaptic activity-response element
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Slc6a3	Solute carrier family 6 protein
SLE	Seizure-like event
SOP	Standard operating procedure
SNP	Single nucleotide polymorphism
SRE	Serum response elements
SRF	Serum response factor
t	Time
TAE	Tris-acetic acid-EDTA buffer
TE	Tris-EDTA buffer
TEMED	tetramethylethylenediamine

T_m	Melting temperature
TOP	5’ Terminal oligopyrimidine
TrkB	Tropomyosin receptor kinase B protein
U	Unit
UoW	University of Waikato
UTR	Untranslated region

Chapter One

Introduction

Anaesthetics, from the Greek anaisthēsia (an- meaning without and aisthēsia meaning sensation), are a range of drugs used during surgery to bring about reversible loss of consciousness, analgesia, areflexia and amnesia. They are paramount in medicine today as they enable the performance of long, painful surgeries without the interference of the patient's pain or reflexes. General anaesthetics were first discovered in 1842 by Crawford Long of Athens, Georgia, USA. Long repeatedly used ether to operate without pain, yet he did not publicise this discovery. General anaesthetics were introduced to the public in 1846 by Dr. William Morton, an American dentist [1, 2]. Morton demonstrated a surgery where a patient was under complete anaesthesia by using the volatile gas, diethyl ether in the correct dose, to a group of influential surgeons at Massachusetts General Hospital, USA. Another commonly used inhalation anaesthetic was nitrous oxide. These early inhalation anaesthetics had many adverse effects on the patients, for example, vomiting and nausea.

In 1932, sodium thiopental was the first intravenous (IV) anaesthetic to be used [3]. This had a larger therapeutic range and less undesirable side effects post operation. Intravenous anaesthetics therefore opened up safer avenues to anaesthesia [4]. In the mid-1900s isoflurane became popular due to the quick recovery from unconsciousness. Isoflurane however was not used to induce anaesthesia, as it commonly irritated throats [5]. Thus, sodium thiopental was initially used to induce the patient, followed by the use of isoflurane to maintain the anaesthesia. In 1971 Dr. Ross Terrell of New York, USA synthesised sevoflurane, which had quick induction times, less chance of throat irritability, fewer cardiovascular depressant effects and slightly faster recovery times [6]. The reduction in throat irritability enabled sevoflurane to be used as an

induction and maintenance anaesthetic. Propofol was first developed in 1976 by Imperial Chemical Industries, London, UK and has largely replaced sodium thiopental as an IV induction anaesthetic due to patients having a more rapid recovery [4]. Although propofol is most often used for induction, it can also be used for maintenance.

For over a hundred years general anaesthetics have been used to eliminate pain during surgery, yet little is known about their biochemical pathways or how they produce amnesia. Brain activity during anaesthesia can be recorded using an electroencephalogram (EEG). This does not show the molecular effects within the brain, or why amnesia is a postoperative side effect. The side effects of anaesthetics can range from something as simple and common as nausea or a sore throat to severe and very rare effects, such as intraoperative awareness or death [7]. One of the most common side effects is amnesia, and just like the molecular pathways of the anaesthetic agents, little is known about how amnesia is brought about. Although amnesia is required for a successful surgery, it becomes a disadvantageous and detrimental post-surgery side effect where it can play a role in postoperative cognitive dysfunction. Intraoperative awareness is when the patient regains consciousness or can recall events during the surgery and is one of the reasons why amnesia is required for surgery. As a result, this can lead to posttraumatic stress disorder (PTSD) surrounding the events of the surgery, thus amnesia is advantageous. Intraoperative awareness is a serious complication with 20,000 to 40,000 of the 4 million surgical patients in the USA being affected [7, 8].

Research into changes in the brain caused by general anaesthetics has predominately investigated the physiological and neurochemistry aspects, which includes blood flow, electrical activity and energy metabolism [9]. With the availability of modern techniques, for instance, microarray and full transcriptome sequencing, it has been shown that from a microarray consisting of 2596 detected transcripts, 417 genes had their expression levels changed

by at least two-fold, in the rat hippocampus, after a clinical dose of the general anaesthetic sevoflurane. The vast majority of genes showed a decrease in expression and over 20% of these were related to cellular metabolism or primary metabolism [10]. Because of this large variety of proteins that are affected, amnesia could be the result of expression changes in memory-related genes.

Clinical dosage levels for the volatile general anaesthetics; isoflurane and sevoflurane vary between 1 – 3% depending on the weight of the patient but also the accompanying gases [11]. Lower doses are needed with a nitrous oxide/oxygen mix while higher doses are needed when using just oxygen. This clinical dose is what is needed for the reversible loss of consciousness, but loss of consciousness is not required for cognitive dysfunction and amnesia. Doses as low as 0.7% isoflurane or 1% sevoflurane have been shown to bring about cognitive dysfunction and memory loss [7]. At these doses the patient may not be completely unconscious and can even be aware of their surroundings. Similar results have been shown with sevoflurane where a clinical dose of between 2 and 2.5 mg/kg is required to induce anaesthesia while memory loss can occur at 1.9 mg/kg [12].

Side effects may not just present themselves during anaesthesia or directly after, but may persist for weeks after anaesthesia is administered. Postoperative cognitive dysfunction (POCD) is a temporary decline in cognitive function over a period of a few days to a few weeks, post operation. The symptoms include amnesia, confusion, reduced awareness, and attention. Postoperative cognitive dysfunction is a growing problem in medicine today, due to an aging population with higher susceptibility of elderly persons [13, 14]. The cause of POCD is unknown, but general anaesthetics have been shown to affect gene expression greatly even 48 hours after anaesthesia, [9]. As a result, expression changes could continue for days or weeks. General anaesthetics have also been shown to reduce the number of synapses in

developing rodents and non-human primates [15, 16]. This is of great importance as pediatric patients that go under anaesthesia for extended periods of time can be affected by anaesthetic neurotoxicity and irreversible damage [17].

This study investigates how two general anaesthetics; propofol and sevoflurane effect gene expression using an *in vitro* mouse model. Primary targets will be candidate memory-related genes, *Bdnf* and *Arc*.

1.1 Memory Consolidation

The mammalian brain is a complex organ involved in information processing, perception, motor control, arousal, homeostasis, motivation and most importantly; memory and learning [18]. The neocortex of the brain has been shown to play a role in memory and learning processes [19].

Communication within the brain occurs between neurons, which consist of a soma, dendrites and an axon [20]. Each neuron has one axon protruding from its soma. The connections between a neuron axon terminal and another neuron's dendrites are called synapses. Synapses are the connection point between the presynaptic neuron axon terminal and the postsynaptic neuron dendrite (Figure 1). Synapses enable neurons to communicate with each other using chemicals as signaling molecules [21]. These molecules are known as neurotransmitters. The axon terminal releases neurotransmitters, and dendrites have receptor proteins that recognise the different neurotransmitters. There are excitatory and inhibitory neurotransmitters. Glutamate is often the excitatory neurotransmitter and binds to the N-methyl-D-aspartate (NMDA) receptor or the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. Gamma-aminobutyric acid (GABA) is the inhibitory neurotransmitter and binds to the GABA receptors [22, 23].

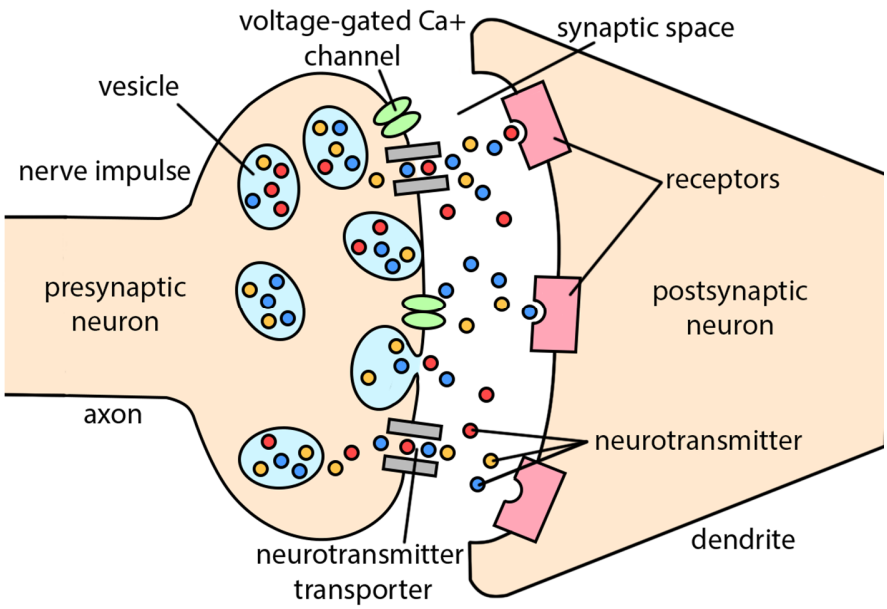


Figure 1: Synapse between Two Neurons. Diagram shows the neurotransmitters (red, yellow, blue circles), their transporters and receptors of the presynaptic neuron (left) and postsynaptic neuron (right) [24].

Neurons are able to communicate with each other by using action potentials which are propagated along the axon and received by a synapse [23]. An excitatory signal results from a flow of positively charged ions into the neuron or negatively charged ions out of the neuron. Inhibitory signals are the opposite; a flow of negatively charged ions into the neuron or positively charged ions out of the neuron. For a neuron to pass an excitatory signal to another neuron it must reach a net excitation before the signal will travel down the axon. Excitatory and inhibitory synaptic signals therefore act against each other and a neuron must receive enough excitatory signals to propagate the signal. However, if the neuron also receives inhibitory signals, the net excitation may not be high enough to continue the synaptic excitation. This is how the brain reacts and decides what to do after receiving information from many different stimuli. When both the presynaptic and postsynaptic neurons are continuously activated, the synaptic connection is strengthened and this is thought to be the basis of memory storage [22, 23].

Memory can be considered as short-term or long-term depending on the brain's ability to recall the information. Short-term memory, or working memory, only lasts 20 to 30 seconds so it must be 'saved' [25]. This process is called consolidation and occurs when there is repetition of a memory. When the same presynaptic neurons are continuously releasing neurotransmitters, while the postsynaptic neuron receptors are being activated, the synaptic connection is strengthened. A synaptic connection can also be strengthened by an increase in the number of postsynaptic neurotransmitter receptors, causing the neuron to respond more efficiently to the neurotransmitters. This is the basis for the synaptic plasticity as a mechanism for memory consolidation [24].

1.2 Synaptic Plasticity and Long-Term Potentiation

Synaptic plasticity is the strengthening or weakening of the synapses from varying levels of activity, whereas, long-term potentiation (LTP) is the continuous re-enforcement of synapses due to current activity levels. Memory is a result from the permanent modification of the synapses between cortical neurons activated by a particular event. Synaptic plasticity and LTP are thought to be the underlying mechanisms for memory consolidation. The biochemical pathway that initiates LTP induction starts with glutamate binding to the NMDA receptors, causing calcium (Ca^{2+}) to enter the neuron, while the postsynaptic membrane is depolarised. The elevated Ca^{2+} levels activate protein kinases which phosphorylate specific substrate proteins, such as AMPA receptors to increase their activity, leading to the enhanced synaptic effectiveness [19, 26]. To gain LTP, a critical level of Ca^{2+} must enter the neuron through the NMDA receptor channel. If this level is not reached then long-term depression (LTD) will occur in the activated synapses. LTD is the opposite of LTP: where LTP is a long lasting signal transmission between two neurons that are stimulated at the same time, LTD is the reduction in efficacy of neuronal synapses. LTD remains stable for many hours without any sign of decay, just like LTP. Therefore, LTD can store information long enough to contribute to hippocampal memory storage [19].

LTP and LTD are perpetuated by synaptic strengthening or synaptic plasticity. This enables the synapses between specific neurons to be strengthened and therefore causes a stronger response to a specific stimuli. Short-term memory was shown to not be consolidated into long-term memory when protein synthesis inhibitors were given to goldfish (*Carassius auratus*) [25]. This supports the hypothesis that synaptic plasticity requires synthesis of *de novo* neurotransmitter receptor proteins. Short-term memory formation still occurred with protein inhibitors present, showing that short-term memory does not require synaptic strengthening. Post-translational protein modification, like the phosphorylation of AMPA receptors for increased activity, is also part of synaptic strengthening. This would still occur with protein synthesis inhibitors, yet memory was still unable to be consolidated. Therefore the synaptic plasticity is not caused from just an increase in receptor proteins or post-translational protein modification of memory-related proteins, but a combination of both [25].

1.3 The Cerebral Cortex

The cerebral cortex is the outer most layer of the mammalian brain and contains the neocortex (Figure 2) [27]. It is grey matter consisting mainly of neuronal cell bodies and capillaries. Under this is white matter, which consists mainly of white myelinated sheaths of neuronal axons; thus, resulting in its white colour. The cerebral cortex folds into bulges called gyri and valleys called sulci. These folds are due to the placement of the neuronal bodies in the cerebral cortex and the axons in the white matter. This increases the surface area, thereby increasing the amount of grey matter for information processing.

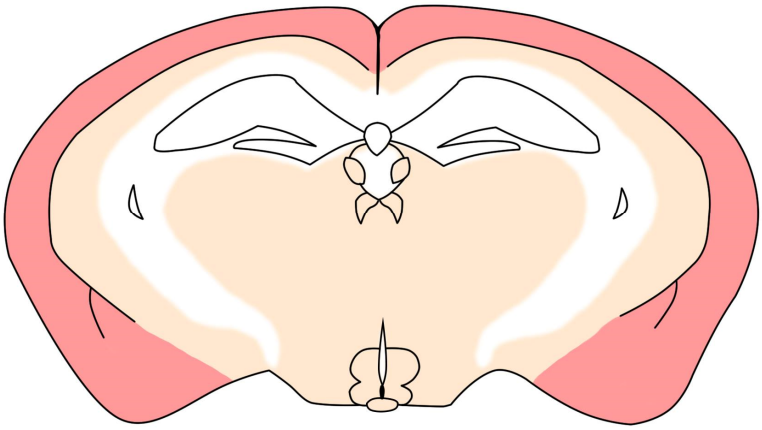


Figure 2: Cerebral Cortex on a Coronal Section of a Mouse Brain. The cerebral cortex is the outermost layer of the brain (red).

The neocortex is the largest part of the cerebral cortex, spanning the two cerebral hemispheres, and is differentiated into six layers; I-VI. These layers were first described by Korbinian Brodmann in 1909, however Santiago Ramón y Cajal was awarded the Nobel Prize in Physiology or Medicine in 1906 for his work in the structure of the nervous system including the cerebral cortex (Figure 3) [18, 28]. Layer I consists predominately of dendrites from pyramidal neurons, glial cells and axons from stellate cells; whereas, Layer II contains mostly small pyramidal and stellate cells. Layer III has larger pyramidal neurons with axons that reach all the way down into the white matter. Layer IV is made up of closely packed stellate cells and, also in this layer a large number of horizontally oriented fibers form a white band. Layer V is composed of large pyramidal cells with few stellate cells and another band of horizontal fibers. Finally, layer VI consists of pyramidal cells again, but with a fusiform shape, which are large in the middle and taper off at both ends. The cerebral cortex is most notably involved in short-term memory. However, the medial temporal lobe and prefrontal cortex has been strongly associated with long term memory consolidation [29].

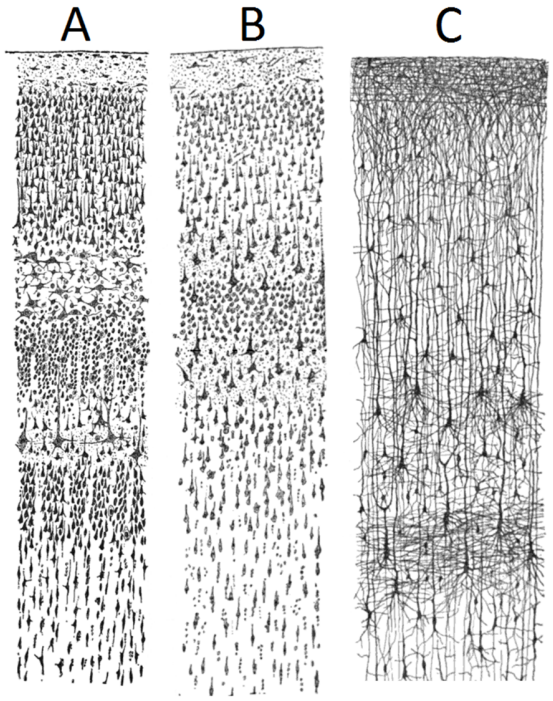


Figure 3: Drawing of the Layers of the Cerebral Cortex by Santiago Ramón y Cajal. A) Nissl-staining (endoplasmic reticulum staining) of the visual cortex of a human adult. B) Nissl-stained motor cortex of a human adult. C) Golgi-stained (dendrites and cell soma staining) cortex of a 1 ½ month old infant [30].

1.4 Physicochemical and Pharmacodynamics of General Anaesthetic Agents

1.4.1 Propofol

Propofol (2,6-di(propan-2-yl)phenol) is a phenolic derivative general anaesthetic, but it is not structurally related to other barbiturates – the classic class of central nervous system (CNS) depressant drugs (Figure 4). Propofol is a useful general anaesthetic due to its rapid onset and short duration of action [31]. Because it is a liquid, it is introduced intravenously and can be used by itself to both induce and maintain anaesthesia. Propofol has a dose-dependent effect on patients, ranging from mild sedation to complete general anaesthesia. At low dosage levels, it can produce amnesia and it has

been given the name “milk of amnesia” due to this property [31]. As propofol is highly lipophilic, it is able to penetrate the blood-brain barrier quickly, resulting in its rapid onset. This rapid penetration and amnesic side effect makes propofol a great candidate for studies of gene expression in the brain.

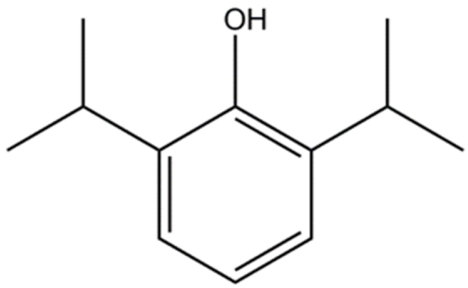


Figure 4: Chemical Structure of Propofol [32].

The mechanism of action for propofol is unknown, but thought to be through the activation of $GABA_A$ receptors, either through direct interaction with the receptor or by modulating $GABA_A$ receptor activity [33]. NMDA receptors are inhibited by propofol, by preventing the movement of Ca^{2+} into neuronal cells. A calcium influx within cells causes the activation of proteases, lipases and endonucleases, which can damage neurons. Propofol can therefore act as a neuroprotective agent by inhibiting the NMDA receptors [32].

Concentrations of 27.5 mg/kg propofol show that LTP is reduced 10-minutes post tetanus stimulation in mouse brain slices of the hippocampus region. This reduced potentiation is also evident immediately after tetanus stimulation, but to a lesser extent. At concentrations of 11 mg/kg and below, this reduction in potentiation does not occur. LTD is not affected at any concentration of propofol. Clinical use of propofol requires a concentration of between 2 and 2.5 mg/kg to induce anaesthesia in humans so the levels of propofol required to affect LTP is up to 25 fold more. However, the majority of this difference would be due to slow perfusion of propofol into brain slice tissue [34]. Only 1.9 mg/kg of propofol is needed for propofol-induced memory loss. Therefore, LTP reduction may not be the cause of memory loss [12].

1.4.2 Sevoflurane

Sevoflurane (1,1,1,3,3,3-Hexafluoro-2-(fluoromethoxy)propane) is a vapour anaesthetic first approved for clinical use in Japan in 1990 [35]. Sevoflurane is used for both the induction and maintenance of general anaesthesia and is a highly fluorinated methyl isopropyl ether (Figure 5). It is a popular general anaesthetic due to its rapid onset and lack of airway irritation [36]. Under anaesthesia, sevoflurane can reduce current times by up to 90%, effectively blocking the effect of GABA on its $GABA_A$ receptor [37]. Sevoflurane has also shown to decrease the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by up to 30%. GAPDH is an enzyme involved in the glycolysis pathway and is directly related to amount of glucose available for breakdown into energy. This reduction in activity prevents conformational changes in GAPDH which are required for catalysis [38].

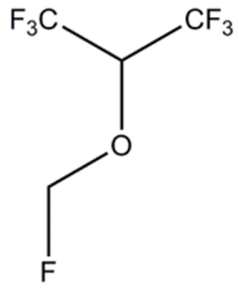


Figure 5: Chemical Structure of Sevoflurane [36].

There has been increasing evidence that sevoflurane impedes memory processes due to the changing of gene expression in neurons in the hippocampus [10]. This impairment can last for at least two days in young rats while up to 10 days in adult rats. Sevoflurane has been shown to up-regulate many different genes, for example, solute carrier family 6 protein (*Slc6a3*). This is a membrane bound transport protein that allows for the movement of solutes across the membrane, either through facilitated transport or coupling to the movement of another solute. *Slc6a3* transports dopamine and it contributes to

different aspects of learning and memory processing. The upregulation of this protein in the hippocampus would cause an increase in the release of dopamine which may result in memory impairment. Acetylcholine receptor alpha-3 (*Chrna3*), a membrane receptor protein that binds the neurotransmitter acetylcholine and transports ions across the membrane is also shown to be up-regulated [10]. The upregulation of the *Chrna3* may be the cause of the unconsciousness associated with sevoflurane.

Sevoflurane has also been shown to down-regulate oxytocin neurophysin (*OT*) and arginine vasopression (*Avp*) which have both been implicated in learning and memory retention, and consolidation [10]. Rat Insulin-like growth factor-1 (*IGF-1*) has been shown to be down-regulated with sevoflurane [39]. Insulin-like growth factor-1 is essential for normal growth and development of the brain, including cell proliferation, cell differentiation and cell survival. Insulin-like growth factor-1 mediates cell survival by preventing apoptosis. A reduction of the *IGF-1* gene has the largest impact on the pyramidal cells of the *cornu ammonis* (CA) regions in the hippocampus.

1.5 Genes of Interest

The genes of interest were chosen based on a pilot study (Laura Bell, UoW; 2014) which observed the differential expression of brain-derived neurotrophic factor (*Bdnf*) and activity-regulated cytoskeleton-associated protein (*Arc*) within the mouse brain *in vivo* after exposure to the two general anaesthetics, propofol and sevoflurane.

1.5.1 Brain-Derived Neurotrophic Factor

Bdnf is the most abundant and widely distributed neurotrophin in the mammalian CNS [40]. The *Bdnf* gene is located on chromosome 2 in mice and consists of one coding exon at the start of the gene, followed by at least eight non-coding exons [40]. In comparison, human *BDNF* gene is on chromosome 11.

Transcribed *Bdnf* mRNA always contains the first coding exon and one of the non-coding exons. The non-coding exon that is selected is determined by the tissue that *Bdnf* is being transcribed in. For example, exon II is only found in the brain while exon III is found in the brain, spleen and kidney [40]. At least nine different promoters are found upstream of the gene, some of which are sensitive to changes in neuronal activity while others are regulated by hormones or epigenetics [41].

Bdnf, just like other neurotrophins, plays a role in neuronal development in foetal humans and mice [42]. Bdnf knockout mice developed with a reduction in CNS neurons and, most notably, had a marked reduction in peripheral neuronal development [43]. This caused the majority of mice to die soon after birth [43]. However, Bdnf does not just help the development of neurons in the foetus, it is also helps differentiate new neurons from neural stem cells and controls apoptosis of neurons [44]. Bdnf is expressed in highest levels in the cortex, hippocampus and basal forebrain, which are all important in learning and memory [45].

Bdnf binds to two known receptors, tropomyosin receptor kinase B (TrkB) and p75 neutrophin receptor ($p75^{NTR}$) [46, 47]. 5' cap mRNAs and 5' terminal oligopyrimidine (TOP) mRNAs are regulated by Bdnf through binding to TrkB and stimulating the phospoinositide 3-kinase (PI3-K) or extracellular signal-regulated kinases (ERK/MAPK) [48]. Basal levels of Bdnf expression cause an increase in protein kinase B (Akt) activity which results in lowered caspase-9 and -3 activity and therefore increasing cell survival [47]. However, general anaesthesia by isoflurane upregulates Bdnf in the cerebral cortex which decreases Akt activity, therefore increasing caspase-9 and -3 activity which leads to apoptotic neurodegeneration (Figure 6) [47].

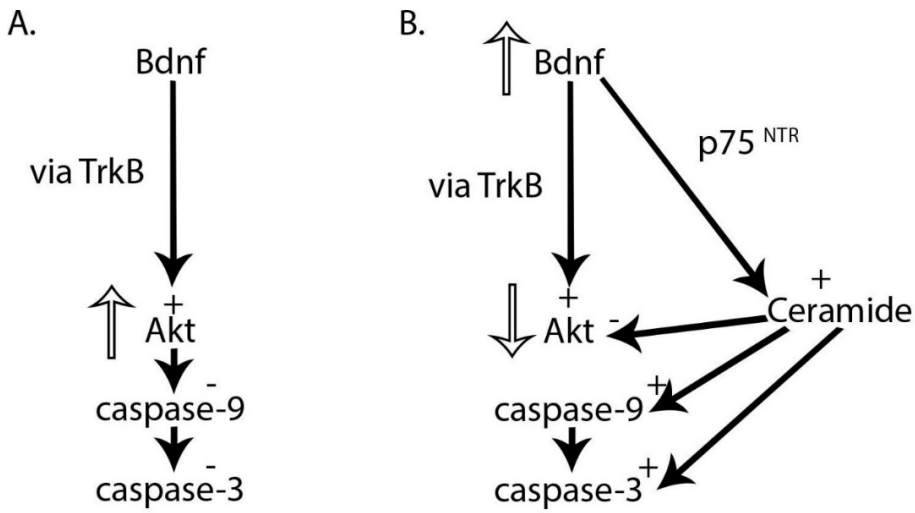


Figure 6: How Bdnf Influences Apoptosis. A) Bdnf is expressed at a basal level and increases Akt activity via TrkB which reduces caspase-3 activity, therefore increasing cell survival. B) Bdnf levels are increased in the cerebral cortex due to isoflurane. However Akt activity is reduced from the $p75^{NTR}$ pathway which overrides the TrkB pathway resulting in high caspase-3 activity and cell apoptosis.

Bdnf has been shown to induce activity dependent translation of dendritically-localised mRNAs, including *Arc*, which are essential in synaptic plasticity [49]. *Bdnf* transcription is required for maintenance of late-phase LTP, therefore may be necessary for memory consolidation and recall [50].

1.5.2 Activity-Regulated Cytoskeleton-Associated Protein

Arc is an immediate early gene (IEG) that is rapidly expressed in neurons following a learning experience or induction of LTP through high-frequency stimulation (HFS) or Bdnf and has a role in altering synaptic strength [51, 52, 53]. This gene is located on chromosome 8 in humans and chromosome 15 in mice. The *Arc* gene consists of three exons and two introns resulting in a 396 amino acid protein with a predicted weight of 55 kDa.

Transcription of *Arc* occurs in response to synaptic activity through the synaptic activity-response element (SARE) which contains binding sites for cyclic AMP response element-binding protein (CREB) and a myocyte enhancer

factor 2 (Mef2) [54]. The *Arc* promoter also contains multiple enhancer elements, three serum response elements (SRE) that bind serum response factor (SRF) which is recruited by synaptic activity or Bdnf [55, 56]. There is a "Zeste-like" enhancer element that is also activated by synaptic activity or Bdnf [56]. Within 1 hour after transcription, *Arc* mRNA can be detected in dendrites up to 300 μm away [57]. Transport to dendrites is accomplished through a heterogeneous nuclear ribonucleoprotein (hnRNP) binding to an 11 nucleotide response element within the coding region of Arc. Kinesin is used as a motor protein to move the *Arc* mRNA along microtubules to the recently activated dendrites where it is locally translated [58, 59]. *Arc* mRNA contains exon junction complexes (EJC) that make it a target for nonsense-mediated decay (NMD), causing quick destabilisation of the mRNA. Also, Arc protein contains a proline, glutamic acid, serine, threonine (PEST) sequence that causes the protein to be quickly degraded by proteasomes, with an estimated half-life of 47 min [60].

Arc antisense (AS) inhibited LTP consolidation but was reversed by F-actin stabilising drug, jasplakinolide. This strongly suggests that Arc's function is in F-actin expansion and stabilisation [61]. Long-term depression induced by dihydroxyphenylglycine (DHPG), a metabotropic glutamate receptor (mGluR) agonist, requires rapid translation of Arc. Inhibition of Arc translation shows a reduction in AMPA receptor endocytosis rates [62]. Therefore Arc translation plays a role in both LTP and LTD [22]. In Arc knockout mice early phase LTP is enhanced while late phase LTP is blocked [63]. Also, disruption of *Arc* expression has led to the impairment in synaptic efficiency and memory consolidation [64]. In addition, Arc was down regulated in response to sevoflurane-induced anaesthesia [65].

1.6 Real-Time Quantitative Polymerase Chain Reaction

Polymerase chain reaction (PCR) was first developed in 1983 by Kary Mullis, who was awarded the 1993 Nobel Prize in Chemistry for his contributions to the developments of methods within DNA-based chemistry [66]. PCR uses a series of steps involving thermal cycling, dideoxynucleotides and DNA polymerase enzyme to amplify single copies of DNA to millions of copies. This is useful in situations where only part of a gene needs to be sequenced, and when only small amounts of a sample are available, such as a hair collected from a crime scene. PCR has three main steps of denaturation, annealing and elongation. During the denaturation step, the thermal cycler heats the samples up to 95 °C to disrupt the hydrogen bonds between the double stranded DNA. This results in single stranded DNA. The temperature is then lowered to 50-65 °C for annealing, based on the temperature of the specific primers being used. The primers bind to their complementary recognition sequence on the single stranded DNA. After this the polymerase, usually *Taq* polymerase which was isolated from the thermophilic bacteria *Thermus aquaticus*, will bind to the primer-template DNA strand. In elongation the temperature is raised to 72 °C to synthesise a new DNA strand from the template strand [67, 68].

Real-time PCR (qPCR) has a very similar method to PCR except it is able to quantify a targeted DNA molecule. This is achieved by using either (1) a non-specific fluorescent dye that binds with all double stranded DNA or (2) an oligonucleotide that is specific to part of the DNA sequence that is being amplified. This has a fluorescent reporter that fluoresces only after hybridisation with its complementary sequence. Quantification through qPCR is only a rough estimate as DNA integrity can be lower in some cases and higher in others, and enzyme efficiency may change. To improve the estimate, standardisation to reference genes is required. Reference genes are constitutive genes required by all cells for basic cellular function and survival. Commonly used standard genes are tubulin, which forms microtubules within

a cell and is present in all eukaryotic cells, or *GAPDH* [69]. The minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines state a minimum of two reference genes is required for accurate quantitative analysis [68].

1.7 Animal model for exposure trials

The C57BL/6 strain of mouse, *Mus musculus*, was used. Mice are considered a good model organism to study human traits. Firstly, mice are small and easy to breed and maintain. For example, the cost of housing a mouse for an experiment is very low compared to other model organisms like non-human primates. A breeding pair can produce around 8-15 pups per litter, once each month. This timeframe allows for genetic inheritance analysis between generations. Secondly, the mouse genome has been completely sequenced and is approximately the same size as the human genome and contains about the same number of genes [70]. In addition, the genome shows great synteny to the human genome. For most human genes, mice have a similar gene and mutations in these genes, often cause the same diseases. Knockout mice are available for many different genes [71].

1.8 Hypothesis, Aims and Objectives

1.8.1 Hypothesis

It is hypothesised that there will be a change in gene expression of the memory-related genes, *Bdnf* and *Arc,* within the cerebral cortex of a mouse brain after a period of anaesthesia.

1.8.2 Aims

The aim of this research is to study the change in gene expression of *Bdnf* and *Arc* receptors in the cerebral cortex of the mouse brain after a period of anaesthesia from sevoflurane and propofol, using qPCR. This research has potential to help discover new anaesthetic drugs or learn more about neurodegenerative disease such as Alzheimer's disease.

1.8.3 Objectives

Objective one is to study the expression of the genes of interest in mouse cortical tissue after *t=4 hour* period of anaesthesia from propofol and sevoflurane. Five mice will be used for each general anaesthetic where each mouse will have three brain slices used; one for treatment, one for *t=0 hour* control and one for *t=4 hour* control. This will be achieved through a range of molecular methodologies. Briefly,

a) preparation of 400 µM mouse brain slices;
b) period of anaesthesia;
c) cortical tissue dissected from each slice;
d) RNA extraction;
e) DNAse treatment;
f) cDNA synthesis; and
g) qPCR.

Objective two is to analyse the qPCR results to determine gene expression changes using the ΔΔCT method.

Objective three is to confirm that the Bdnf protein is present in the cerebral cortex tissue of the mouse brain. This will involve immunohistochemistry (IHC) using commercial Bdnf antibodies. Briefly,

a) preparation of OCT brain slice cryomolds;
b) preparation of 10 µM sections using the Cryostat;
c) staining with hematoxylin and eosin;
d) incubation with primary antibodies and FITC secondary antibody; and
e) imaging microscope slides under UV light on the microscope.

Objective four is to confirm that the antibody is binding specifically to the Bdnf protein using western blotting. Briefly, this will involve:

a) extraction of protein from mouse brain;
b) running the protein extract on a SDS-PAGE gel;
c) transfer of protein to a membrane;
d) binding of primary and secondary antibody to the epitope on the membrane; and
e) imaging the membrane using chemiluminescence.

Chapter Two

Materials and Methods

The majority of these methods were carried out in the Molecular Genetics Laboratory (C.2.03) at the University of Waikato (UoW), Hamilton, New Zealand. The cryosectioning was performed in the Mammalian Physiology Laboratory (E.2.14), the microscopy work in the Microscopy Laboratory (R.2.29) and anaesthetic procedures in the Cortical Research Laboratory (C.1.14). Milli-Q water (mQH_2O) purified at 15-18 MΩ·cm was used for all solutions and experiments. In addition, water used in RNA research was treated with 0.1% diethylpyrocarbonate (DEPC) to inactivate any RNase enzymes that may have been present. Glassware was autoclaved at 120 °C for 15 minutes and dried overnight in an 80 °C oven. Recipes for buffers and solutions used in this study can be found in Appendix 1.

2.1 Statement on Animal Ethics

The method for anaesthetising and euthanising all mice used in this research study was approved by the University of Waikato Animal Ethics Committee (protocol number 905, Appendix 2), and was in compliance with the standards provided in SOP Number: 9 (Appendix 3).

2.2 Brain Tissue Experimentation

To study the effects of anaesthetics on mouse neocortex *in vitro*, whole mouse brain was first extracted from a freshly euthanised mouse. For the anaesthetics to completely perfuse through the tissue the slices needed to be a maximum of 400 µm thick. For both propofol and sevoflurane there was a sample size of *n=5* animals with *t=0 hour* control, *t=4 hour* control and *t=4 hour* treated slices taken from each mouse.

2.2.1 Dissection and Slicing of Brain Tissue

Brain tissue was dissected from 10- to 15-week old C57BL/6 strain mice under carbon dioxide anaesthesia. After the whole brain was removed from the animal, it was immersed in a 100 mL beaker of carbogenated (95% oxygen, 5% carbon dioxide) ice-cold “normal” artificial cerebrospinal fluid (nACSF) (125mM NaCl; 2.5 mM KCl; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 1.25 mM NaH_2PO_4; 26 mM $NaHCO_3$ and 10 mM glucose). One to two millimetres of tissue was cut off the front and the rear of the brain with a scalpel blade to provide a flat surface to glue the brain onto a cutting block. The block was inserted into a vibratome (Campden Instruments, UK) and the tissue was sectioned in ice-cold nACSF into 400 µm thick coronal sections from front to back. Once a section was cut it was placed into carbogenated magnesium (Mg)-deficient ACSF (124 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 1.25 mM NaH_2PO_4; 26mM $NaHCO_3$ and 10 mM glucose). The brain slices were kept in the Mg-deficient ACSF at room temperature (RT) for two hours to activate seizure-like event (SLE) activity in the cortical tissue. Use of Mg-deficient ACSF is a widely used technique for generating SLE activity in cortical slices [72].

2.2.2 Confirming Viability of Brain Sections

Viability of the neocortical tissue was determined by use of an extracellular microelectrode (50 µm Teflon-coated tungsten) measuring local field potential (LFP) SLE activity. A section was placed into a perfusion bath (Kerr Tissue Recording System, Kerr Scientific Instruments, Christchurch, New Zealand) containing Mg-deficient ACSF perfused at a rate of 5 mL/min. The electrodes were sequentially placed into six different locations in the neocortex and spontaneous electrical activity measured (Figure 7). The signal was amplified (250x), band-pass filtered (high pass 1 Hz; low pass 100 Hz) and sampled at 1000 Hz (PowerLab, ADInstruments, Sydney, Australia). The presence of Mg-deficient SLE activity (Figure 8) was used as an indication that the tissue was viable. In particular, the criterion that a minimum of 4 of 6 recorded locations showed SLE activity was used to include a slice in the study. This

was an arbitrary threshold based on the desire to use only those slices where the majority of the tissue could be shown to be viable. SLE activity was also used to infer the action of the anaesthetic on the brain slice as SLE activity is effectively blocked by volatile and non-volatile general anaesthetic drugs [72]. Seizure-like event electrical activity was used to generate a box and whisker plot to compared activity between the propofol and sevoflurane slices.

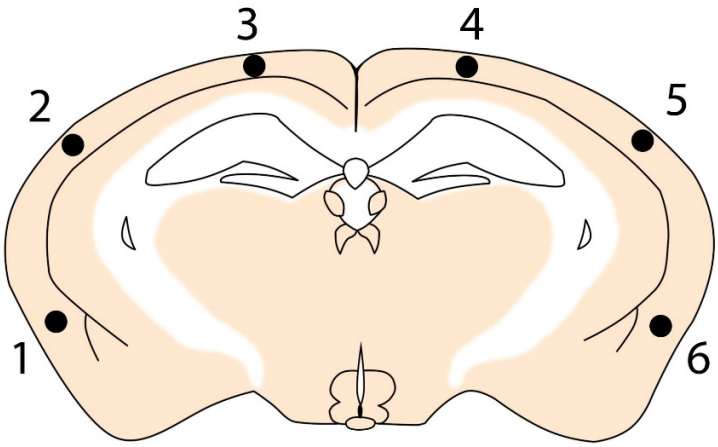


Figure 7: Electrical Impulse Recording Sites of Brain Slice. Brain slice showing the six locations measured for electrical impulses.

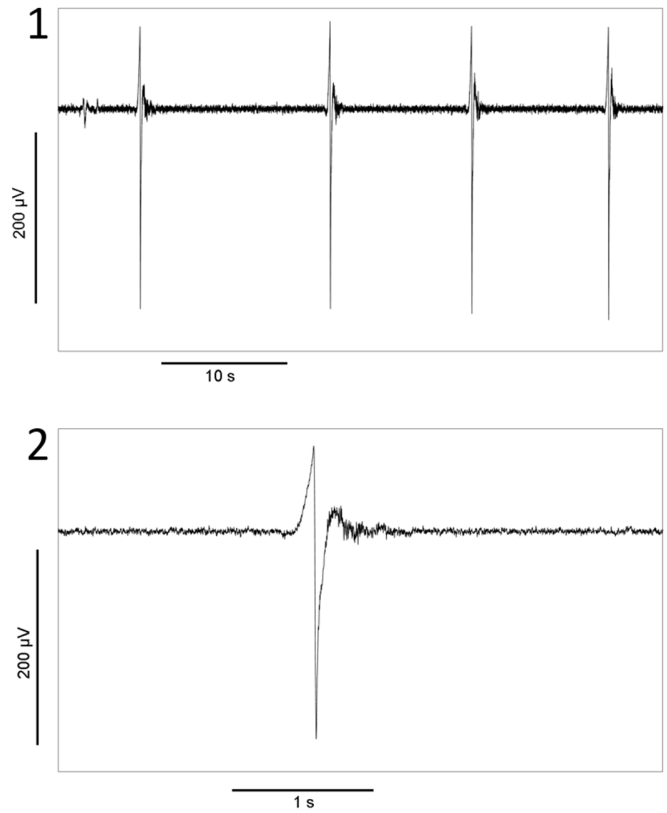


Figure 8: Example of SLE Activity. 1) SLE activity over a short period of time. 2) Enlarged activity graph.

2.2.3 Propofol Anaesthetic Procedure

Propofol stock was prepared as a 1% solution of 2,6-di(propan-2-yl)phenol 97+% (SAFC supply solutions, USA) in 20% Intralipid (Fresenius Kabi AB, Sweden). A final propofol concentration of 10 µg/mL was made by mixing 0.2 mL of stock solution into 200 mL of Mg-deficient nACSF. After a viable section was identified, a net was placed on top of the slice to prevent flotation, remove surface tension and prevent the slice from drying out. The propofol solution was placed into a gravity fed drip system with the flow rate set to 5 mL/min. SLE activity was recorded over the *t*= 4 hour treatment to observe the decline in SLE's as the propofol took effect. Untreated control brain slices were kept in carbogenated nACSF for the duration of the anaesthetic exposure and then the neocortex was extracted for RNA.

2.2.4 Sevoflurane Anaesthetic Procedure

With a viable slice prepared as described above in section 2.2.2, sevoflurane was delivered in carbogenated Mg-deficient ACSF using a syringe pump-driven system run at a flow rate of 5 mL/min. A final concentration equivalent to one minimum alveolar concentration (MAC) of sevoflurane was delivered to the brain tissue by adding 0.02 mL of liquid sevoflurane to 50 mL nACSF in the sealed syringe. The amount of sevoflurane required to reach one MAC using this method was determined by a previous study that showed through HPLC and headspace gas analysis that isoflurane reached one MAC when 0.01 mL was added to 50 mL nACSF in a syringe and pumped at 5 mL/min [73]. Sevoflurane is used at 2.1% in oxygen for anaesthesia while isoflurane is used at 1.1%. Therefore, sevoflurane is half as potent as isoflurane so the amount added to the nACSF was doubled from the isoflurane experiments to reach one MAC sevoflurane [5, 11]. Similar to the propofol anaesthetic procedure, the SLE activity was recorded over the *t*=4 hour treatment to observe the decline in activity and ensure the sevoflurane was taking effect. Control slices were kept as described above in section 2.2.3.

2.2.5 Neocortex Extraction

A sterile scalpel was used to cut the neocortex away from the subcortical tissue in the slice. This tissue was placed into a sterile 2 mL screw cap tube containing 1 mL of TRI Reagent® (Sigma-Aldrich, USA), a quarter of a cap of 0.1 mm zirconia/silica beads and one 2.5 mm glass bead. Immediately after extraction, the tissue samples were lysed using a FastPrep® FP120 (Thermo Scientific, USA) speed beater at a speed of 6.5 for 25 seconds at RT. This was repeated three times to ensure all the cells were completely lysed with a two minute interval between each cycle to prevent overheating of the machine and sample. The TRI Reagent® prevented the degradation of RNA and DNA. RNA was then extracted from the lysate.

2.3 RNA Extraction and Quantification

RNA extraction was carried out on a dedicated bench for RNA work that had been decontaminated with RNase AWAY® (Invitrogen™, USA). These procedures minimise RNase contamination. All centrifugation was carried out in an Eppendorf 5424R centrifuge (Eppendorf, Germany) at 12,000 x g at 4 °C. The lysate containing glass beads was centrifuged for two minutes to collect the beads and cell debris at the bottom of the tube and the supernatant was transferred to a new 2 mL tube with 200 µL of chloroform for protein removal. After 15 seconds of vigorous shaking the samples were placed on a rotating wheel for 15 minutes at RT and then centrifuged for a further 15 minutes. The aqueous phase was transferred to a new 2 mL tube with 500 µL of isopropanol. The samples were allowed to stand at RT for 10 minutes to precipitate the RNA. The samples were centrifuged for eight minutes and the RNA formed a pellet on the side of the tube. The supernatant was removed from the tube and the pellet washed with 1 mL of 75% ethanol to remove salts. A final five minute centrifugation was done to ensure the RNA pellet was still on the side of the tube before removing the ethanol. After a brief air dry to remove the remaining ethanol, the RNA pellet was dissolved in 20 µL of 0.1% diethylpyrocarbonate (DEPC) treated water at 65 °C for 15 minutes and then cooled on ice for five minutes before quantification and storage.

2.3.1 Quantity and Quality of RNA

RNA quality and quantity was determined using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, UK). The NanoDrop measured the optical density at certain wavelengths (230 nm, 260 nm and 280 nm) to calculate the quantity and quality. An A_{260}/A_{280} ratio of 1.8-2.0 is accepted as "pure" for RNA and an A_{260}/A_{230} ratio of 2.0-2.2 for low phenol or guanidine contamination. RNA samples were stored at -80 °C to reduce RNA degradation over time. Each RNA sample was labelled according to its treatment (P, S), replicate number (1-5) and time point (0C, 4C, 4).

2.3.2 Native RNA Gel Electrophoresis

RNA samples were run on a 1% native agarose gel to analyse the RNA integrity by viewing the ribosomal RNA bands (18S and 28S). To prepare the gel, 0.4 g of HyAgarose™ agarose (HydraGene, USA) was dissolved into 40 mL of 1X TAE (40 mM Tris; 20 mM acetic acid; and 1 mM Ethylenediaminetetraacetic acid (EDTA)) buffer. This was conducted in a microwave and boiled until the solution was clear and free of agarose particles. After the agarose was completely dissolved and cooled to 60 °C, 1.5 µL of 10 mg/mL ethidium bromide (EtBr) was mixed into the solution. The gel was then poured into a level 8 cm x 7 cm gel mold and a comb was inserted. Once the gel had set, 4 µL of RNA (200-400 ng/µl) was mixed with 1 µL of loading buffer and loaded onto the 1% agarose gel. Gel electrophoresis was electrophoresis at 45 V for one hour in 1X TAE buffer and the resulting gel was imaged on the Omega Lum G gel imager (Aplegen, USA).

2.3.3 DNAse Treatment

The RNA sample was DNAse-treated before the synthesis of complementary DNA (cDNA) in order to remove any genomic DNA contamination that was still present after RNA extraction. A 10 µL reaction was prepared in a 0.2 mL microcentrifuge tube which contained DNAse I enzyme (Zymo Research, USA), 1 µg of RNA sample, 10X DNAse Buffer and DEPC water (Table 1). This reaction was heated at 37 °C for one hour in a PTC-200 Peltier Thermocycler (Geneworks, Australia) to degrade DNA and then 1 µL of 500 mM EDTA was added to chelate Mg ions and heated at 65 °C for 10 minutes to inactivate the DNAse I enzyme. DNAse-treated RNA was stored at -80 °C until required for cDNA synthesis.

Table 1: DNAse Reaction Components. Each reaction was made up in a 0.2 mL microcentrifuge tube.

Component	Quantity (µL)
10X DNAse buffer	1
RNA sample	1 - 8 (1 µg of total RNA)
DEPC water	Up to 10 µL
DNAse I enzyme	1

2.3.4 Complementary DNA Synthesis

DNA polymerase used in PCR can only amplify up double stranded DNA (dsDNA); therefore, the mRNA samples must first be converted to cDNA. A 20 µL reaction was prepared in a 0.2 mL microcentrifuge tube containing Tetro Reverse Transcriptase (Bioline, USA), RiboSafe RNase Inhibitor, 10 mM dNTP mix, 5X reverse transcriptase buffer, random hexamer primers, DNAse-treated RNA, and DEPC water (Table 2). Random hexamer primers were selected to provide uniform coverage rather than creating bias at the 3' end using Oligo(dT) primers. A negative reverse transcriptase control was used where the reverse transcriptase was replaced with DEPC water. This was done to ensure the DNAse procedure worked and that there was no genomic DNA contamination in the sample following PCR.

Table 2: cDNA Synthesis Reaction Components. Each 20 µL reaction was made up in a 0.2 mL microcentrifuge tube

Component	Concentration	Quantity (µL)	Final Concentration
RNase inhibitor	10 U/µL	1	0.5 U
dNTP mix	10 mM	1	0.5 mM
Reverse transcriptase buffer	5X	4	1X
Random Hexamers	40 µM	1	2 µM
DNAse-treated RNA		1	N/A
DEPC water		11	N/A
Reverse transcriptase	200 U/µL	1	10 U

The samples were incubated in a PTC-200 Peltier Thermocycler (Geneworks, Australia) to carry out the reaction. The reaction program is outlined in Table 3.

Table 3: cDNA Synthesis Program. Synthesis of cDNA from RNA using these conditions.

Cycle	Temperature (°C)	Time (min)
Activation	25	10
Reverse transcription	45	30
Heat inactivation	85	5

2.4 PCR Sample Testing

PCR was carried out to ensure the synthesised cDNA could be amplified with no evidence of genomic DNA contamination as this would skew the qPCR results. In the PCR hood, a master mix was prepared in a 1.5 mL microcentrifuge tube with HOT FIREPol® DNA polymerase (Solis BioDyne, Estonia). The master mix was then aliquoted out into 0.2 mL microcentrifuge tubes and cDNA added. The reaction mixture is outlined in Table 4 and standard PCR primers used in Table 5.

Table 4: Reaction Components of PCR. Each reaction was made up in a 0.2 mL microcentrifuge tube

Component	**Concentration**	**Quantity (µL)**	**Final concentration**
B2 buffer	10X	2	1X
$MgCl_2$	25 mM	1.2	1.5 mM
dNTPs	10 mM	0.4	200 µM
Forward Primer	10 µM	0.5	0.25 µM
Reverse Primer	10 µM	0.5	0.25 µM
DEPC water		14.2	N/A
cDNA		1	N/A
HOT FIREPol® Polymerase	5 U/µL	0.2	0.5 U/µL

Table 5: Six Primers Used in Standard PCR. Primer sequences used for standard PCR to test amplification of the cDNA samples. Two housekeeping genes were used, *β2m* and *Gapdh*. *β2m* primers amplified up an exon-exon boundary while *Gapdh* did not. Therefore *Gapdh* was used to verify no genomic DNA contamination. *Bdnf* was used to check the presence of a gene of interest. Genbank accession numbers are provided [74].

Gene	Primer Sequence (5' – 3')	Product (bp)	Accession Number
β2m	F: TTCTGGTGCTTGTCTCACTGA	104	NM_009735.3 [75]
	R: CAGTATGTTCGGCTTCCCATTC		
Gapdh	F: TGCACCACCAACTGCTTAGC	87	NM_001289726.1 [76]
	R: GGCATGGACTGTGGTCATGAG		
Bdnf1	F: TGAGTCTCCAGGACAGCAAA	103	NM_007540 [77]
	R: GCCTTCATGCAACCGAAGTA		
Bdnf2	F: GTGACAGGCGTTGAGAAAGC	206	NM_007540 [77]
	R: ATCCACCTTGGCGACTACAG		
Arc	F: GGTGAGCTGAAGCCACAAAT	104	AF162777.1 [78]
	R: GCTGAGCTCTGCTCTTCTTCA		
HPRT1	F: TGACACTGGCAAAACAATGCA	94	NM_013556.2 [79]
	R: GGTCCTTTTCACCAGCAAGCT		

Samples were amplified according to the program outlined in Table 6. Amplification was performed on a T100™ Thermal Cycler (BioRad, USA). For each PCR run there were two controls. Firstly a negative control to ensure the PCR components were free of DNA contamination. This negative control contained 1 µL of DEPC water instead of template cDNA. Secondly, reverse transcriptase negatives were used to ensure the cDNA was free of genomic DNA.

Table 6: PCR Program. All end-point PCR reactions were done in 0.2 mL PCR microcentrifuge tubes and followed this program.

Step	Temperature (°C)	Time	Cycle
Initial denaturation	95	15 minutes	1x
Denaturation	95	20 seconds	30x
Annealing	60	30 seconds	
Elongation	72	40 seconds	
Final elongation	72	5 minutes	1x

Samples were run on 2% agarose gels stained with EtBr for band visualisation under UV light as outlined in section 2.3.2. Five microliters of PCR DNA Ladder (GenScript, USA) was run with the samples to estimate the size of the PCR products.

2.5 Quantitative Polymerase Chain Reaction (qPCR)

5X HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Estonia) was used for qPCR and the reactions were carried out according to the optimised program recommended by the manufacturer. A mastermix was made to obtain the final concentrations required for qPCR. These are outlined in Table 7.

Table 7: qPCR Mastermix Components. Mastermix was made up in a 1.5 mL microcentrifuge tube before being pipetted out into the 0.2 mL qPCR microcentrifuge tubes.

qPCR Master Mix Components	Concentration	Quantity (µL)	Final Concentration
DEPC water		5	N/A
Forward primer	10 µM	0.5	250 nM
Forward primer	10 µM	0.5	250 nM
5X HOT FIREPol® EveGreen® qPCR Mix Plus	5X	4	1X

The cDNA samples were diluted 1:20 with DEPC water and, for each reaction, 10 µL of cDNA was added. Adding 10 µL of cDNA for each reaction reduced the variation between the duplicates in the qPCR analysis. Each qPCR run contained all the *t*= 4 hour treatment and time-matched controls and was run with one primer at a time. The samples were run on a Rotor-Gene 6000™ Thermocycler (QIAGEN, Germany) using a 36 tube rotor disc. Every sample was run in duplicate with a negative control which contained no cDNA. A final melt curve was prepared to indicate how many qPCR products were present. One product should be present for each primer. The qPCR program is outlined in Table 8.

Table 8: qPCR Program. All qPCR reactions were done in 0.2 mL qPCR microcentrifuge tubes and followed this program which was optimised by Solis Biodyne. The samples were run on the Rotor-Gene 6000™ Thermocycler in the 36 well rotor disc.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	12 minutes	1x
Denaturation	95	15 seconds	40x
Annealing	65	30 seconds	
Elongation	72	30 seconds	
Final melt	80	10 seconds	

2.6 Statistical Analysis

The Rotor-Gene™ 6000 software produces raw quantitative data which produces C_T values based on a threshold set by a user. These C_T values were applied to the $\Delta\Delta C_T$ method. The $\Delta\Delta C_T$ equation is outlined in Equation 1. The $\Delta\Delta C_T$ method compares the amplification of a specific primer in the treated sample and control sample. The fold changes generated from this comparison are then normalised to multiple housekeeping genes to give a normalised fold change of the genes of interest (GOI). Microsoft Office Excel (Microsoft, USA) was used to analyse the qPCR results and generate fold change and relative expression graphs.

Equation 1: $\Delta\Delta C_T$ Method for qPCR Analysis.

$$\Delta C_{T(treatment)} = \text{Gene of Interest } C_{t(treatment)} - \text{Reference gene } C_{t(treatment)}$$

$$\Delta C_{T(control)} = \text{Gene of Interest } C_{t(control)} - \text{Reference gene } C_{t(control)}$$

$$\Delta\Delta C_T = \Delta C_{T(treatment)} - \Delta C_{T(control)}$$

Quantitative PCR data shows the changes in mRNA expression levels; therefore, protein levels are also expected to change. Western blots and immunohistochemistry analysis was used to examine protein level changes. A two-tailed Student's t-test was used to calculate a p-value that showed whether or not a result was of statistical significance.

2.7 Organ Dissection and Protein Extraction

Six organs (brain, heart, lung, kidney, spleen, and liver) were dissected from C57BL/6 strain mice between 10 and 15 weeks of age. The organs were washed with ice-cold 1X PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 at pH 7.4) and placed individually into 2 mL screw cap tubes containing one capful of 0.1 mm zirconia/silica beads, eight 2.5 mm glass beads and 500 µL of cell lysis buffer with protease inhibitor (Complete Mini Protease Inhibitor Cocktail Tablet (Roche, Germany)). The tissue samples were lysed using a FastPrep® FP120 (Thermo Scientific, USA) speed beater at a speed of 6.5 for 25 seconds at RT. This was repeated three times with a two minute interval between each cycle. The total cell lysates were then centrifuged for one minute at 12,000 x g to pellet cellular debris and glass beads. The supernatant was transferred to a new tube to remove the glass beads and cell debris. The cell lysate samples containing proteins were stored at -80 °C until required for the Bradford assay.

2.7.1 Bradford Assay on Cell Lysates

The Bradford assay is an accurate procedure used to determine the concentration of protein in a solution. The Bradford dye is made up by mixing one part of Bio-Rad Protein Assay reagent (BioRad, USA) with four parts mQH_2O and then filtering using Whatman® #1 filter paper. The Bradford dye was stored at 4 °C until used. A 10 mg/mL stock solution of bovine serum albumin (BSA) (Sigma-Aldrich, USA) was prepared and standards were diluted with 1X PBS as outlined in Table 9. The stock and working solutions were stored at -20 °C.

Table 9: BSA Standards Concentrations. Concentrations of the BSA standards used in the Bradford Assay

	Concentration of standard (mg/mL)									
	0	0.1	0.25	0.5	1	2	4	6	8	10
10 mg/mL BSA (µL)	0	1	2.5	5	10	20	40	60	80	100
1X PBS (µL)	100	99	97.5	95	90	80	60	40	20	0

A 96 well flat bottom, non-treated microplate (JET BIOFIL, China) was used for the assay and 100 µL of Bradford dye reagent was pipetted into each well of a row to be used followed by the addition of 1 µL of the appropriate BSA standard. The sample was mixed using a pipette tip. The organ cell lysates were diluted to 1:100 to bring the protein concentrations into the accurate readable range and then 1 µL was pipetted into wells separate to the standards that contain 100 µL of Bradford dye reagent. After a 5 minute RT incubation, the plate was inserted into a Multiskan™ GO Microplate spectrophotometer (Thermo Scientific, USA) and the 595 nm wavelength absorbance readings were recorded. Each standard and sample was performed in duplicate. A standard curve was produced and the absorbance values of the cell lysates were compared to this curve to calculate the protein concentrations of each organ tissue cell lysate.

2.8 Western Blot Analysis

Western blot analysis was carried out to validate the antibody that was being used in immunohistochemistry as well as to assess the level of Bdnf protein within whole mouse brain extracts.

2.8.1 SDS-PAGE Gel Electrophoresis

As the anti-BDNF antibody recognises a 28 kDa Bdnf immature protein a 12% resolution sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) was chosen for the western blots. The gel was cast in a Mini-PROTEAN® 3 casting system (BioRad, USA). Milli-Q water was mixed with 3M Tris pH8.8, 10% SDS, 37:1 (22%) Acrylamide:Bisacrylamide, 10% ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) in the volumes outlined in Table 10. The stacking gel recipe is outlined in Table 11.

Table 10: 12% Resolution SDS-PAGE Components. Protein gels were made in a Mini-Protean® III casting system.

Component	Concentration	Quantity (µL)	Final Concentration
Milli-Q water		4485	
Tris pH8.8	3 M	2000	0.4 M
37:1 Acylamide:Bisacrylamide	22%	8200	12%
10% SDS	10%	150	0.1%
10% APS	10%	150	0.1%
TEMED		15	

Table 11: SDS-PAGE Stacking Gel.

Component	Concentration	Quantity (µL)	Final Concentration
Milli-Q water		2985	
Tris pH6.8	0.5 M	1250	0.125 M
37:1 Acylamide:Bisacrylamide	22%	660	2.9%
SDS	10%	50	0.1%
APS	10%	50	0.1%
TEMED		5	

Once the gel had set it was loaded into Mini-Protean® III (BioRad, USA) gel electrophoresis tank with Tris-glycine SDS (25 mM Tris-Cl; 250 mM glycine and 0.1% SDS) buffer. The organ cell lysates were mixed in a 1:1 ratio with 2X sample loading buffer containing 2-mercaptoethanol and heated at 95 °C for 10 minutes in a Thermomixer® (Eppendorf, Germany) to denature the proteins. A 10 µL volume of each sample was loaded into different wells. A 2.5 µL volume of PAGE-MASTER protein standard ladder (GenScript, USA) and 1.25 µL of WB-MASTER protein standard ladder (GenScript, USA) were also added to the gel. Electrophoresis was carried out at 140 V for 55 minutes at RT.

2.8.2 Protein Transfer to PVDF Membrane

Once gel electrophoresis had finished, the gels were removed from the plates and rinsed briefly in mQH_2O. Hybond™-P polyvinylidene difluoride (PVDF) (GE Life Science, USA) membrane was cut to the appropriate size to cover the gel and then soaked in methanol for one minute followed by one minute in eBlot™ equilibrium buffer. The eBlot® system (GenScript, USA) was used to transfer the protein from the gel to the western blot membrane. The system was run for 8 minutes to ensure all protein transferred. Ponceau S stain was used to determine if the protein transfer was successful. After observation of protein bands, the membrane was washed in TBS-T buffer (50 mM Tris-Cl; 150 mM NaCl; 0.1% Tween-20 at pH 7.5) before moving onto the western blot protocol.

2.8.3 Incubation with Blocking Buffer and Antibodies

All steps during this protocol were conducted at RT with gentle agitation on an orbital mixer unless stated otherwise. Fresh 10% blocking buffer was made using milk powder (Alpine Diary Products, New Zealand) in 1X TBS (50 mM Tris-Cl; 150 mM NaCl at pH 7.5). The membrane was blocked for 1 hour using 10% blocking buffer. Following this, the blocking buffer was removed and the membrane washed with 1X TBS-T for 5 minutes. A 1:100 concentration primary antibody solution was made by mixing 10 µL of primary antibody in 990 µL of 10% blocking buffer. The membrane was sealed in a plastic bag with the primary antibody solution overnight at 4°C on an orbital shaker. Primary antibodies used in this research project are outlined in Table 12.

Table 12: Primary and Secondary Antibodies Used in the Western Blot.

Target	Host	Colonity	Concentration	Manufacturer (Country) and Name
Bdnf	Rabbit	Polyclonal	1:100	Santa Cruz Biotech (USA) N-20
Bdnf	Rabbit	Polyclonal	1:100	Abcam (UK) ab72439
Actβ	Rabbit	Polyclonal	1:1000	Genetex (USA) GTX110564
Gapdh	Mouse	Monoclonal	1:1000	Genetex (USA) GTX627408
Goat Anti-rabbit HRP	Goat	Polyclonal Secondary IgG	1:5000	Abcam (UK) ab97051

The next day the primary antibody was removed and the membrane washed with 1X TBS-T three times for five minutes. A 1:10,000 concentration anti-rabbit horse radish peroxidase (HRP) secondary antibody (Abcam, UK) solution was diluted in 10% blocking buffer. The membrane was incubated in this secondary antibody solution for one hour. After incubation the antibody solution was removed and the membrane was washed with 1X TBS-T three times for 15 minutes and a final wash for one hour. After the final wash, the washing buffer was removed and the membrane was developed with 0.1 mL/cm^2 WesternBright™ Sirius™ HRP developing solution (Advansta, USA). This developing reaction was incubated for five minutes in the dark before being imaged on the Omega Lum G gel imager (Aplegen, USA).

2.9 Immunohistochemistry and Microscopy

Hematoxylin and Eosin (H&E) staining procedure was carried out to ensure cells were intact for IHC and to have a reference to navigate the IHC sections. Immunohistochemistry was used to verify that Bdnf was expressed in the neocortex and to compare the level of expression to other regions of the brain such as the hippocampus. Frozen brain tissue was sectioned using a cryostat in preparation for H&E and IHC.

2.9.1 Cryosectioning of Brain Slices

Active brain slices were placed into cryosection molds and covered with FSC 22™ Frozen Section Compound (OCT) (Lecia, Germany) then flash frozen with Frostbite® (Lecia, Germany) spray. The frozen brain slice molds were wrapped in tin foil and kept at -80 °C overnight to ensure they were completely frozen. The Lecia CM1850 UV cryostat machine (Lecia, Germany) chamber was kept at -35 °C during cryosectioning as this temperature produced the best sections. Sections (10 µm) were cut using a Surgipath DB80 LX Premium Low Profile disposable blade (Lecia, Germany) and placed onto Polysine™ microscope slides (LBSP4981) (Thermo Scientific, USA) which were kept in a slide box at -80 °C until used.

2.9.2 Hematoxylin and Eosin Staining

All steps during this protocol and the IHC protocol were done at RT unless stated otherwise. Slides containing brain sections were defrosted for five minutes before surrounding the sections with Liquid Blocker (Super Pap Pen) (Daido Sangyo, Japan) to reduce the amount of H&E used. After the pap pen had dried, the slides were fixed in 4% paraformaldehyde (PFA) for five minutes and then washed with running tap water for one minute to remove excess formaldehyde. Surgipath® Harris Hematoxylin (Lecia, Germany) stain was pipetted onto the slides, on a horizontal staining tray, and left for five minutes to stain the nuclei and then washed in water to remove excess stain. Scott's tap water was used as blueing reagent to help hematoxylin blue the nuclear chromatin and nuclear membranes. The slides were then rinsed with Scott's tap water for two minutes. Surgipath® Eosin (Alcoholic) (Lecia, Germany) was pipetted onto the slides and left for ten minutes to stain red the negatively charged proteins in the cytoplasm. The slides were washed with running water three times for three minutes each time and then dried and mounted with Shandon™ Immu-Mount™ (Thermo Scientific, USA). The dry slides were then viewed using an Axiostar plus transmitted light microscope (Carl Zeiss, Germany).

2.9.3 Immunohistochemistry

Slides containing brain sections were defrosted for five minutes before fixing in chilled 4% PFA for 10 minutes and then washed three times for five minutes each time in 1X PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 at pH 7.4). The slides were soaked in 0.5% Triton X-100 for 30 minutes to permeate the cell membranes and then washed three times for five minutes each time in 1X PBS. Slides were soaked in 0.9% H_2O_2 to block endogenous peroxidases and washed two times for five minutes each time in 1X PBS and one time in 1X PBS-T (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4 and 0.2% Tween-20 at pH 7.4) for five minutes. The sections were encircled with Super Pap Pen and left to dry for

two minutes before being washed in 1X PBS. The sections were blocked with 5% goat serum in 0.3 M glycine and washed with 1X PBS for five minutes before pipetting a 1:200 concentration primary antibody solution, which was made by mixing 5 µL of primary antibody in 995 µL of 5% goat serum with 0.3 M Glycine. Glycine was used to quench any unreacted aldehydes therefore reducing background noise. The primary antibody incubated overnight in a humidity box at 4 °C and then washed two times for five minutes each time in 1X PBS and one time in 1X PBS-T for five minutes to remove excess primary antibody. A 1:2000 concentration secondary antibody solution was made using 1 µL of secondary antibody in 1,999 µL of 1X PBS. The secondary antibody was pipetted onto the slides and left for 30 minutes in the dark. Primary and secondary antibodies are outlined in Table 13. A final wash of two times for five minutes each time in 1X PBS and one time in 1X PBS-T for five minutes was done, to remove excess secondary antibody and reduce background, before drying the slides and mounting with coverslips using Fluoroshield™ with DAPI (4', 6-diamidino-2-phenylindole) (Sigma-Aldrich, USA). DAPI binds to adenine and thymine rich regions in DNA and is used to stain the nucleus. The fluorescent slides were viewed using an Axiostar plus transmitted light microscope (Carl Zeiss, Germany). Alexa Fluor ® 488 secondary antibody was excited under 495 nm wavelength light.

Table 13: Primary and Secondary Antibodies Used in IHC.

Target	Host	Colonity	Concentration	Manufacturer (Country) and Name
Bdnf	Rabbit	Polyclonal	1:100	Abcam (UK) ab72439
Goat Anti-rabbit Alexa Fluor® 488	Goat	Polyclonal Secondary IgG	1:2000	Abcam (UK) ab150077

Chapter Three

Results

This chapter outlines the results from the molecular methodologies used to extract RNA from the mouse cortical brain tissue and convert it to a cDNA template, which was subsequently used to analyse gene expression during a four hour anaesthetic drug exposure. The protein data from the western blots and immunohistochemistry on coronal brain slices is also included in this chapter.

3.1 *In Vitro* Anaesthetic Exposure Using a Mouse Model

Adult male and female wildtype C57BL/6 mice between 10 and 15 weeks old were involved in this study. After the cortical tissue was confirmed to contain electrical activity, control slices were kept in nACSF and treatment slices were exposed to either propofol or sevoflurane.

3.2 Brain SLE Activity

Seizure-like events were recorded as a way of identifying brain slices that pertained to our criteria of a viable brain slice. This meant that four of the six recorded locations showed SLE activity (Figure 7). This data shows the SLE frequency and amplitudes which were averaged out across all active locations within a slice and a box and whisker plot generated from these averages for both sevoflurane. These graphs are shown below in Figures 9 and 10.

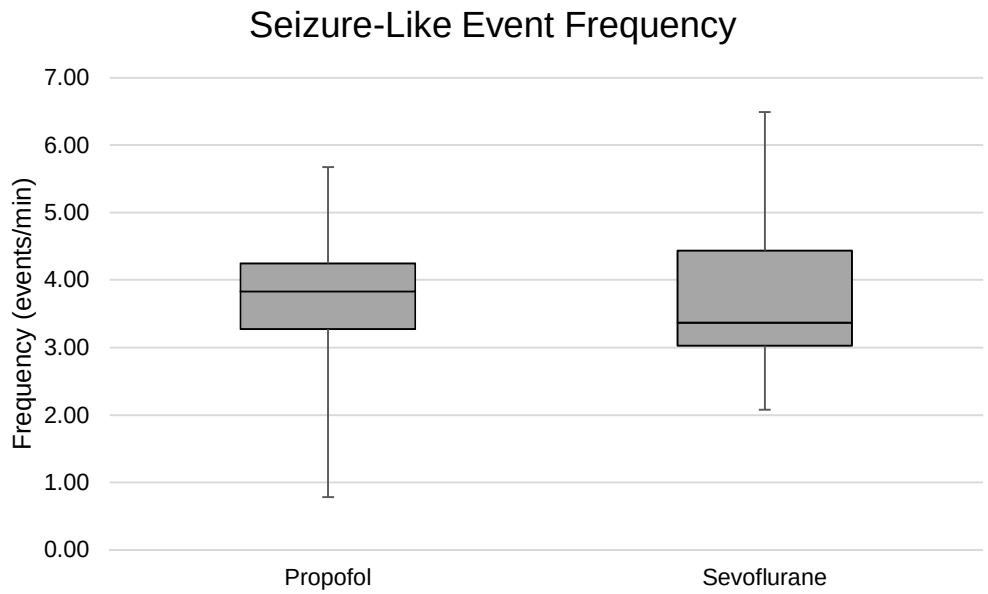


Figure 9: SLE Frequency for Propofol and Sevoflurane. SLE frequency is similar between propofol and sevoflurane, however propofol did have a much lower minimum frequency of 0.78 events/min compared to sevoflurane's 2.08 events/min.

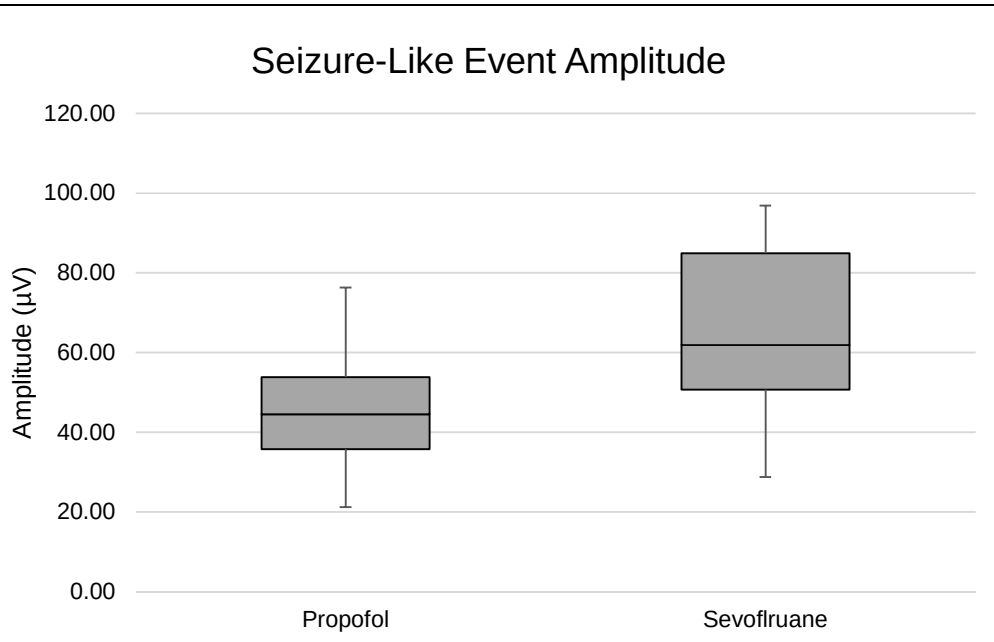


Figure 10: SLE for Propofol and Sevoflurane. SLE amplitude for sevoflurane is increased compared to propofol. The average amplitude for propofol was 44.55 µV while sevoflurane was 61.90 µV.

3.3 RNA Quantification and Qualification

Following confirmation of active electrical recordings, RNA was extracted from the brain slice and the quality and quantity was analysed using a NanoDrop™ 2000 Spectrophotometer. The quantity was assessed so approximately the same amount of RNA template would be used for downstream applications.

3.3.1 NanoDrop Analysis of Propofol RNA Samples

Fourteen of the extracted RNA samples had a NanoDrop reading consistent with pure RNA, except for P5T4C. The RNA concentrations ranged from 193-440 ng/µL (Table 14).

Table 14: Mice Used in the Propofol Treatment Experiment. Mice were aged between 10 and 15 weeks old. Both female and male mice were used. Each RNA sample was labelled according to its treatment (P, S), replicate number (1-5) and time point (0C, 4C, 4).

Sample	Age (Weeks)	Sex	RNA Conc (ng/µL)	A_{260}/A_{280}
P1T0C	10	Female	224.9	1.91
P1T4C			347.9	1.92
P1T4			210.5	1.89
P2T0C	11	Male	373.8	1.87
P2T4C			391.3	1.85
P2T4			301.6	1.88
P3T0C	14	Male	267.9	1.95
P3T4C			226.1	1.88
P3T4			440.3	1.89
P4T0C	15	Male	279.7	1.96
P4T4C			291.5	1.84
P4T4			341.9	1.94
P5T0C	15	Female	310.4	1.94
P5T4C			193.7	1.73
P5T4			290.3	1.87

3.3.2 NanoDrop Analysis of Sevoflurane RNA Samples

All 15 sevoflurane treated and control RNA samples had A_{260}/A_{280} ratios that would be considered pure. The sevoflurane RNA concentrations ranged from 164.1-350.4 ng/µL. Compared to the propofol samples, the sevoflurane samples had a lower average concentration. S5T0C had a RNA concentration that was lower than the rest which could have been from not removing enough of the aqueous layer during TRI Reagent® RNA extraction or from the RNA pellet drying out and not dissolving into mQH_2O (Table 15).

Table 15: Mice Used in the Sevoflurane Treatment Experiment. Mice were aged between 10 and 14 weeks old. Both female and male mice were used. Each RNA sample was labelled according to its treatment (P, S), replicate number (1-5) and time point (0C, 4C, 4).

Sample	Age (Weeks)	Sex	RNA Conc (ng/µL)	A_{260}/A_{280}
S1T0C	10	Female	293.6	1.86
S1T4C			350.4	1.93
S1T4			298.9	1.95
S2T0C	11	Male	262.9	1.86
S2T4C			226.8	1.91
S2T4			242.5	1.80
S3T0C	12	Female	336.5	1.92
S3T4C			251.2	1.86
S3T4			235.8	1.89
S4T0C	13	Male	270.4	1.87
S4T4C			275.7	1.92
S4T4			215.5	1.90
S5T0C	14	Female	164.1	1.85
S5T4C			253.6	1.92
S5T4			225.0	1.87

3.3.3 Non-Denaturing Gel Electrophoresis Analysis of 18S and 28S rRNA for Propofol

To quickly check the integrity of the RNA, two samples were run on a 1% native TAE agarose gel; the original 2000 ng extracted RNA, and 200 ng of DNAse-treated RNA (Figure 11).

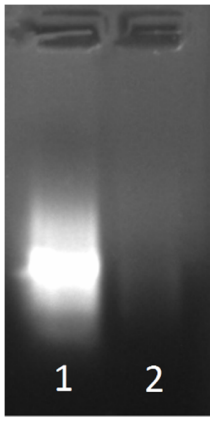


Figure 11: Non-Denaturing Agarose Gel Testing rRNA Integrity. Non-denaturing 1% TAE agarose gel electrophoresis at 45 V for one hour. 1) 2000 ng untreated RNA sample 2) 200 ng DNAse-treated RNA sample. The rRNA bands are not distinguishable and have smeared across the gel. This indicates the RNA has been degraded. A ladder was not included with this experiment.

Figure 11 shows observation of a smear with a bright band in Lane 1. In comparison, the DNAse-treated sample had a lower concentration of RNA so the smear did not show up as clearly as the untreated sample. However, there was no clear indication of rRNA bands. Because the brain tissue was placed directly into Tri Reagent® and extracted immediately, the most likely cause of degradation was the quality of the water used to dissolve the extracted RNA.

To overcome this issue, mQH_2O was treated with 0.1% DEPC followed by sterilisation by autoclaving, and then another RNA sample was extracted from a mouse cortical brain tissue. The sample was then run on a 1% TAE agarose and two distinct bands were present, marking the 18S and 28S rRNA. Therefore, our data demonstrates that the RNA was mostly free of degradation (Figure 12) and suitable for downstream qPCR analysis.

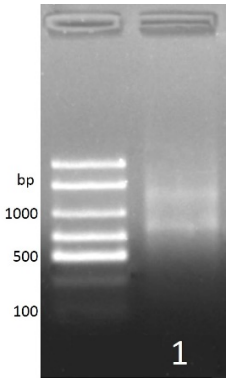


Figure 12: Second rRNA Integrity Test. Non-denaturing 1% TAE agarose gel electrophoresis at 45 V for one hour. 1) 1000 ng of RNA sample after the water had been DEPC-treated and RNA had been DNAse-treated. GenScript® PCR DNA Ladder is included. Both the 18S and 28S rRNA bands are visible in this RNA sample. This indicates that the RNA has not been degraded.

Only the zero hour control (*t=0*) propofol RNA samples were run on a 1% TAE agarose gel to check for degradation due to these samples not being used for qPCR analysis. This was done to minimise the loss of RNA from the samples that were going to be used for qPCR analysis because of low extracted RNA quantity. The zero hour control propofol RNA samples are shown in Figure 13.

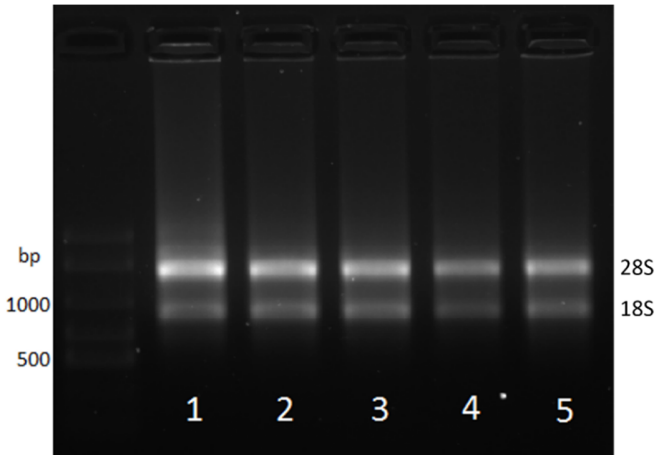


Figure 13: rRNA Bands of the Five *t=0 Hour* Control Propofol Samples. Non-denaturing 1% TAE agarose gel electrophoresis at 45 V for one hour. 1) P1T0C 2) P2T0C 3) P3T0C 4) P4T0C 5) P5T0C. GenScript® PCR DNA Ladder is included.

Both the 18S and 28S rRNA bands can be seen. The 28S band is roughly twice the intensity of the 18S band which indicates good quality RNA.

3.3.4 Non-Denaturing Gel Electrophoresis Analysis of 28s and 18s rRNA for Sevoflurane

As with the propofol samples only the zero hour control sevoflurane RNA samples were run to minimise loss of the RNA samples that were going to be used in qPCR analysis. The zero hour control sevoflurane RNA samples are shown in Figure 14.

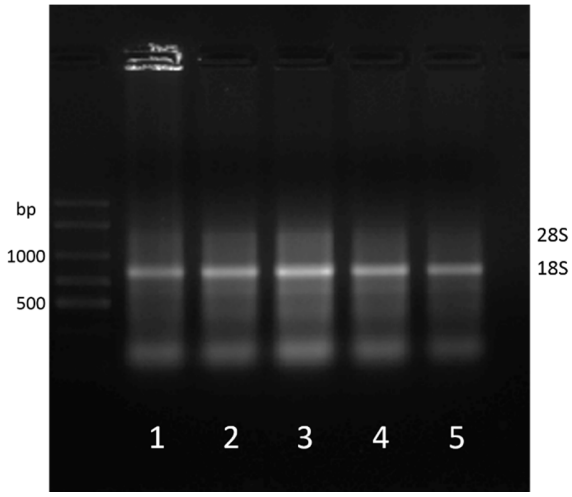


Figure 14: rRNA Bands of the Five *t=0 Hour* Control Sevoflurane Samples. Non-denaturing 1% TAE agarose gel electrophoresis at 45 V for one hour. 1) S1T0C 2) S2T0C 3) S3T0C 4) S4T0C 5) S5T0C. GenScript® PCR DNA Ladder is included.

All 28S and 18S rRNA bands can be seen. However, the 28S bands are faint and not twice the intensity of the 18S bands. This results indicate degradation of RNA.

3.4 Standard PCR to Ensure No Genomic DNA Contamination

After cDNA synthesis, standard PCR was used to determine if any genomic DNA contamination was present in the RNA sample and optimize cycling conditions if necessary. For each sample the -RT control from the cDNA synthesis was tested with the *Gapdh* primer. The *Gapdh* primers produce an 87 bp product but they do not span an exon-exon boundary. As a result, genomic DNA will be amplified if it is present. Thus, the –RT control is expected to contain no cDNA because no reverse transcriptase was included during cDNA synthesis. Alongside the -RT control was the cDNA sample amplified with *Gapdh*, *Bdnf1* (103 bp), *Bdnf2* (206 bp) or *β2m* (104 bp) primers. The *β2m* primer is an exon boundary spanning primer which would only amplify cDNA and so it was used as a positive control to ensure cDNA synthesis occurred without genomic DNA contamination.

3.4.1 Propofol Samples

DNAse-treatment occurred at 37 °C for 15 minutes. After this the DNAse-treated RNA was converted to cDNA and run through a standard PCR test. The agarose gel electrophoresis results from the five P1 amplified samples are shown in Figure 15.

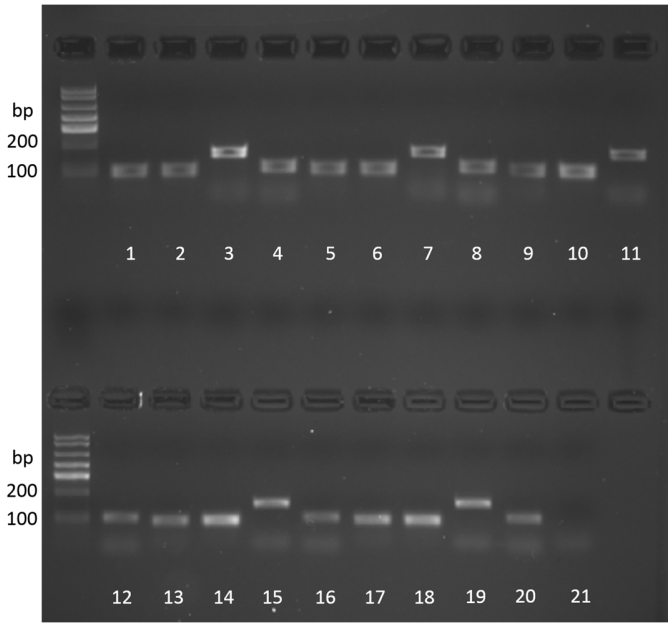


Figure 15: Standard PCR on 15 Minute DNAse-Treated Propofol Samples. This 2% agarose gel contains the P1 cDNA sample set and two test samples and was run at 90 V for 30 minutes. 1) P1T0C -RT control with *Gapdh* primers. 2) P1T0C cDNA with *Gapdh* primers. 3) P1T0C cDNA with *Bdnf2* primers. 4) P1T0C cDNA with *Bdnf* primers. 5) P1T4C -RT control with *Gapdh* primers. 6) P1T4C cDNA with *Gapdh* primers. 7) P1T4C cDNA with *Bdnf2* primers. 8) P1T4C cDNA with *Bdnf* primers. 9) P1T4 -RT control with *Gapdh* primers. 10) P1T4 cDNA with *Gapdh* primers. 11) P1T4 cDNA with *Bdnf2* primers. 12) P1T4 cDNA with *Bdnf* primers. Wells 13 to 20 are protocol test samples. Well 21 is the PCR negative. GenScript® PCR DNA Ladder is included on left-hand side. There is contamination in every -RT control indicating genomic DNA contamination.

The agarose gel showed that there was a band in all the -RT controls and no band present in the PCR negative. This suggested there was genomic DNA contamination in these samples and the DNAse treatment had failed. DNAse treatment was then performed at 37 °C for 30 minutes to optimise genomic DNA removal. The results of the P1 samples with the 30 minute treatment are shown in Figure 16.

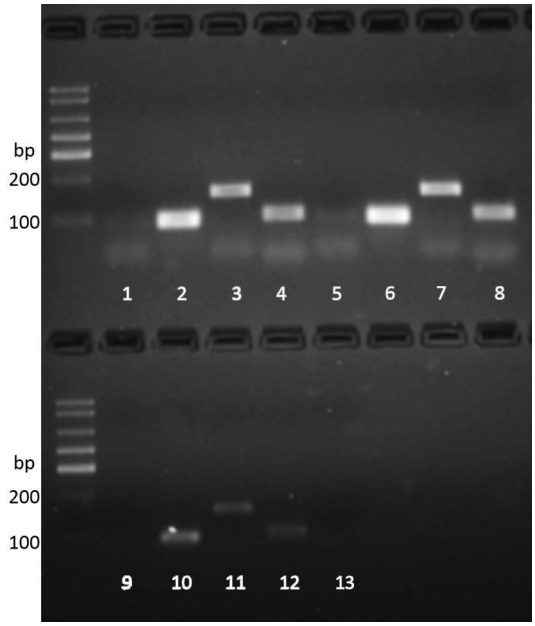


Figure 16: Standard PCR on 30 Minute DNAse-Treated Propofol Samples. 2% TAE agarose gel electrophoresis at 90 V for 30 minutes. This gel contains the P1 cDNA sample set. 1) P1T0C -RT control with *Gapdh* primers. 2) P1T0C cDNA with *Gapdh* primers. 3) P1T0C cDNA with *β2m* primers. 4) P1T0C cDNA with *Bdnf* primers. 5) P1T4C -RT control with *Gapdh* primers. 6) P1T4C cDNA with *Gapdh* primers. 7) P1T4C cDNA with *β2m* primers. 8) P1T4C cDNA with *Bdnf* primers. 9) P1T4 -RT control with *Gapdh* primers. 10) P1T cDNA with *Gapdh* primers. 11) P1T cDNA with *β2m* primers. 12) P1T cDNA with *Bdnf* primers. 13) PCR negative. GenScript® PCR DNA Ladder is included on left-hand side. There is contamination in both the P1T0C and P1T4C -RT control indicating genomic DNA contamination.

Again there was still observation of genomic DNA contamination in the -RT controls. The DNAse treatment time was further increased to one hour. The results from the P1 samples are shown in Figure 17.

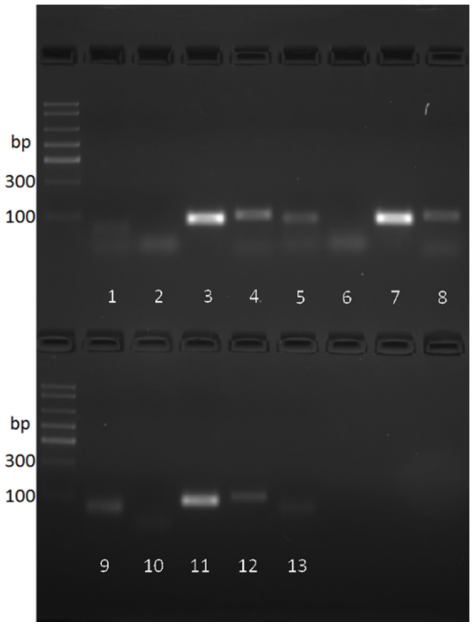


Figure 17: Standard PCR on 60 minute DNAse-Treated Propofol Samples. 2% TAE agarose gel electrophoresis at 90 V for 30 minutes. This gel contains the P1 cDNA sample set. 1) PCR negative. 2) P1T0C -RT control with *Gapdh* primers. 3) P1T0C cDNA with *Gapdh* primers. 4) P1T0C cDNA with *β2m* primers. 5) P1T0C cDNA with *Bdnf* primers. 6) P1T4C -RT control with *Gapdh* primers. 7) P1T4C cDNA with *Gapdh* primers. 8) P1T4C cDNA with *β2m* primers. 9) P1T4C cDNA with *Bdnf* primers. 10) P1T4 -RT control with *Gapdh* primers. 11) P1T4 cDNA with *Gapdh* primers. 12) P1T4 cDNA with *β2m* primers. 13) P1T4 cDNA with *Bdnf* primers. GenScript® PCR DNA Ladder is included on left-hand side. This gel shows there is no genomic DNA contamination within the samples.

The one hour DNAse treatment removed genomic DNA completely from the RNA samples. The *β2m* and *Bdnf* still amplified the expected product size so the long DNAse treatment time did not degrade the mRNA. This PCR protocol was repeated for all five propofol animal sample sets which included the *t=0 hour* control, *t=4 hour* control and *t=4 hour* treatment.

3.4.2 Sevoflurane Samples

The one hour DNAse treatment protocol consistently removed all genomic DNA from the sevoflurane samples as shown in Figure 18.

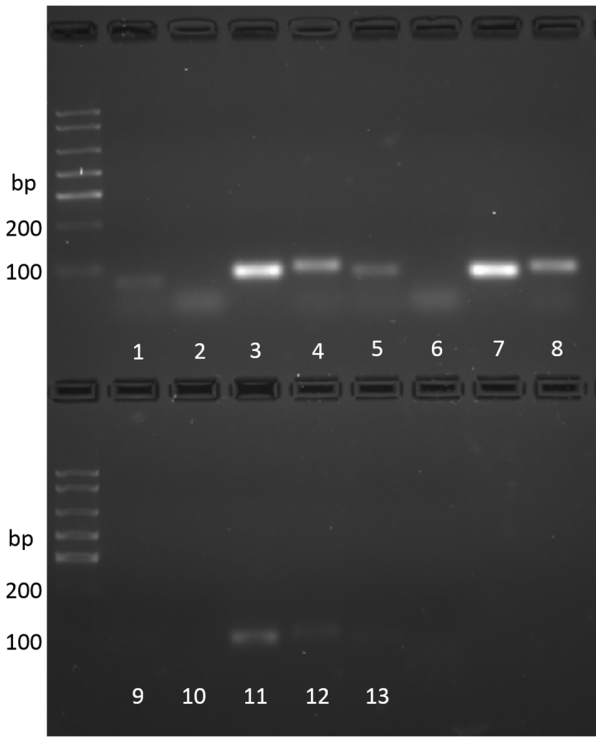


Figure 18: Standard PCR on Sevoflurane Samples. 2% TAE agarose gel electrophoresis at 90 V for 30 minutes. This gel contains the S1 cDNA sample set. 1) PCR negative. 2) S1T0C -RT control with *Gapdh* primers. 3) S1T0C cDNA with *Gapdh* primers. 4) S1T0C cDNA with *β2m* primers. 5) S1T0C cDNA with *Bdnf* primers. 6) S1T4C -RT control with *Gapdh* primers. 7) S1T4C cDNA with *Gapdh* primers. 8) S1T4C cDNA with *β2m* primers. 9) S1T4C cDNA with *Bdnf* primers. 10) S1T4 -RT control with *Gapdh* primers. 11) S1T4 cDNA with *Gapdh* primers. 12) S1T4 cDNA with *β2m* primers. 13) S1T4C cDNA with *Bdnf* primers. GenScript® PCR DNA Ladder is included on left-hand side. This gel shows there is no genomic DNA contamination within the samples.

A band was observed above the primer dimer in the PCR negative which indicated contamination. However, no contamination was seen in any of the three –RT controls therefore it was assumed that this was contamination from pipetting and not contamination within the PCR mix.

3.5 Quantitative PCR Analysis

Quantitative PCR analysis was carried out on the *t=4 hour* control and *t=4 hour* treated samples for both propofol and sevoflurane. *Bdnf* and *Arc* were the genes of interest analysed. Reference genes were used as internal controls to normalise the qPCR results. The MIQE guidelines recommends the use of at least two reference genes [68]. Four reference genes were used in this qPCR analysis; *Gapdh*, *β2m*, *Actβ* and *HPRT1* since they have been used in previous published studies [69, 79, 80]. The nucleotide sequence of the six genes used in this study was previously confirmed by cloning the PCR fragments and Sanger Sequencing (Laura Bell, UoW, NZ; 2014)

3.5.1 Testing Sample Concentrations for qPCR Analysis

There was very little sample to work with in qPCR so diluting the cDNA samples was the only way to ensure enough volume to complete the qPCR analysis. A test qPCR was run using an undiluted sample and one that had been diluted 1/5. *β2m* was amplified and the take-off value used to determine the best dilution to use. If the take-off value was greater than 30 cycles for each sample at the 1/5 dilution then the samples were only diluted by 1/2. The amplification curve for the propofol samples is shown in Figure 19.

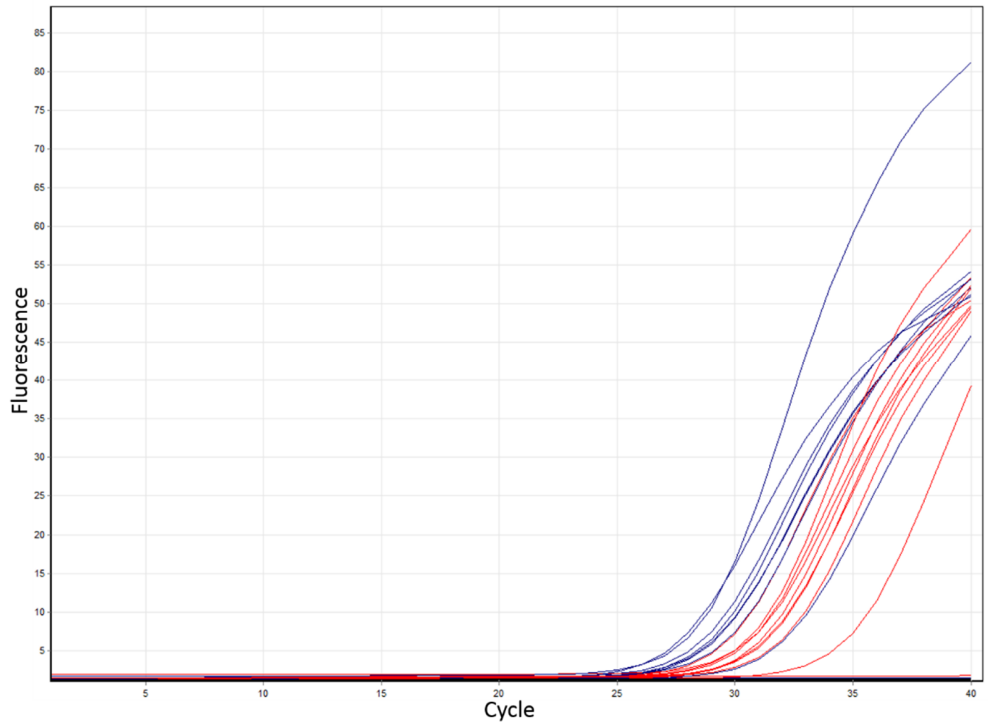


Figure 19: Propofol Sample Concentration Test. The blue lines are propofol samples that have not been diluted. The red lines are the same samples with a 1/5 dilution.

P5T4 with a 1/5 dilution was an outlier having a take-off value of 31.5. P3T4 with a 1/5 dilution failed to amplify but the undiluted sample had a take-off value of 20.9 which is in line with the other undiluted samples so it was assumed that this was due to a loading error and not a sample error. The majority of 1/5 diluted propofol samples have a take-off value lower than 30 cycles so the propofol samples were diluted 1/5. This test was repeated for sevoflurane and the amplification curve for the sevoflurane samples is shown in Figure 20.

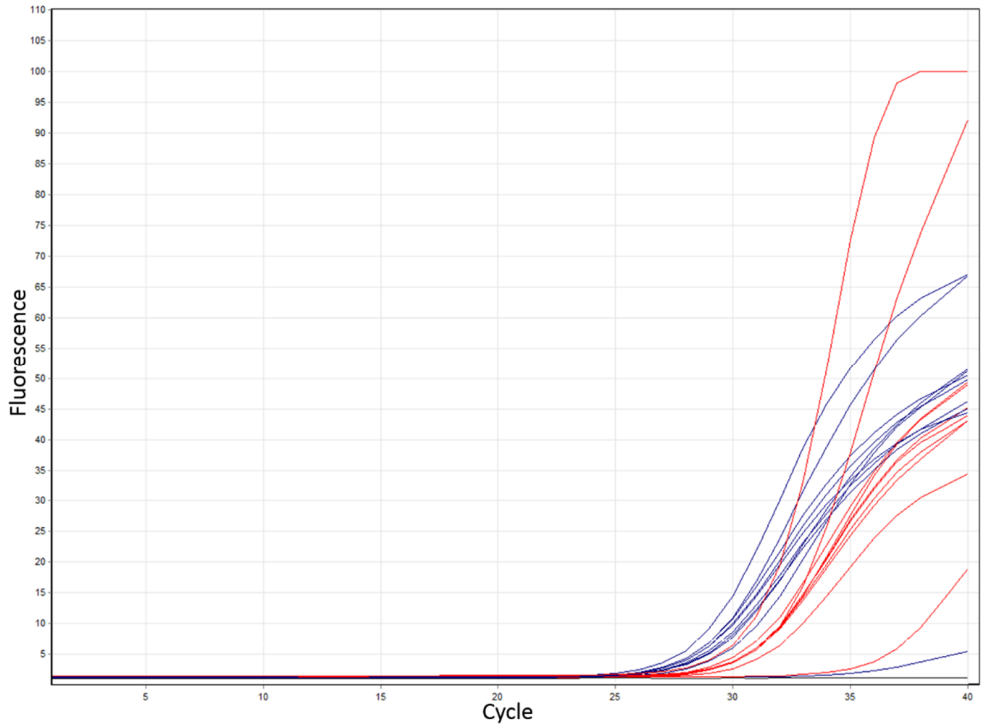


Figure 20: Sevoflurane Sample Concentration Test. The blue lines are propofol samples that have not been diluted. The red lines are the same samples with a 1/5 dilution.

P1T4C undiluted had a take-off value of 31.7 and P1T4C 1/5 dilution had a take-off value of 33.1. This sample was kept undiluted for gene expression analysis. As with the propofol samples, the majority of 1/5 diluted sevoflurane samples have a take-off lower than 30 cycles so the sevoflurane samples were diluted 1/5 and used for gene expression analysis.

3.5.2 Amplification and Melting Point Curves

Each qPCR run contained only one primer set and all samples of either the propofol or sevoflurane sample sets. This was done to remove the run to run variation within each sample set and allow more accurate analysis. Each run had all samples run in duplicates to give more accurate results, and had a no template control which contained the PCR master mix and primer. An example of the amplification graph produced from a qPCR run is shown in Figure 21. All amplification curves are included in Appendix 4.

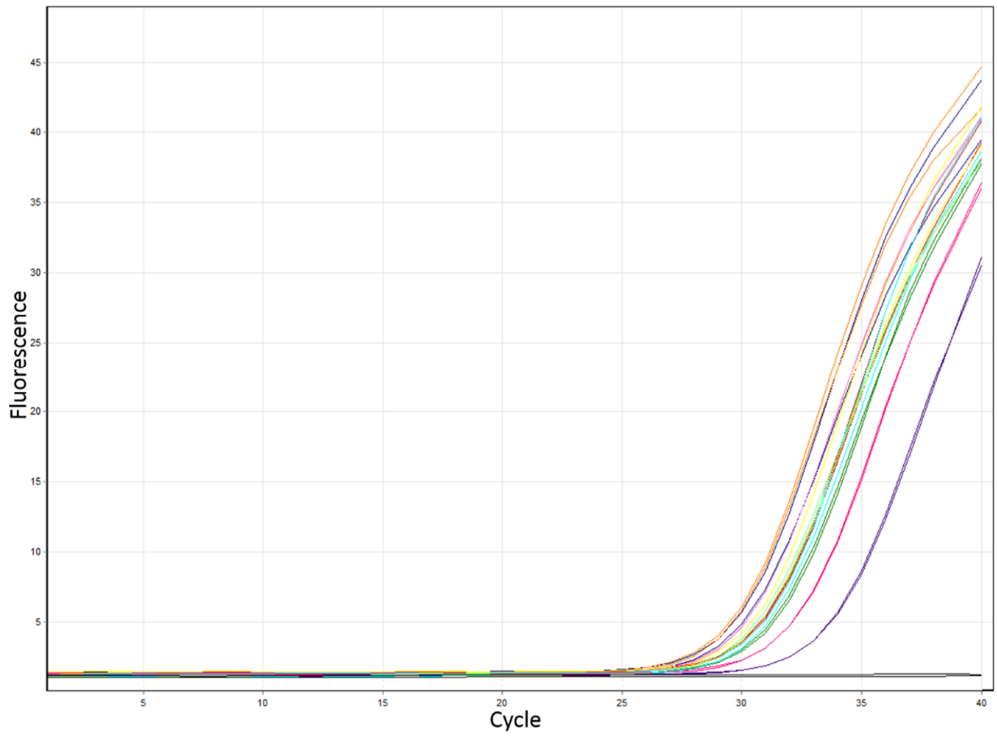


Figure 21: Amplification Curve of *Arc*. Each sample is run in duplicate with only one primer per qPCR run. There are 10 different samples on this curve. Each sample was run in duplicate.

Melt curves were carried out on each qPCR run to analyse the product(s). This ensured that each sample had the same product and that each negative did not contain the same product as the samples, if there was amplification. The temperature at which an amplification product denatures depends on the size of the product and the G-C content. If a melt curve showed that there was the

same product in both the negative and the samples, then this would be considered as contamination and the run would be repeated. The melt curve for *Arc* is shown in Figure 22.

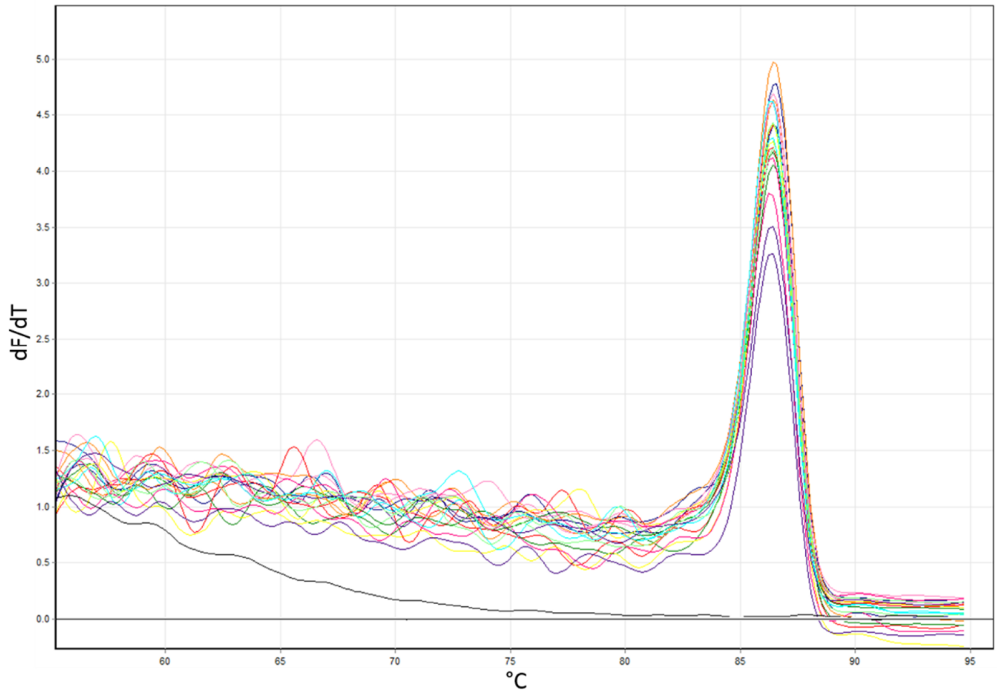


Figure 22: Melting Curve of *Arc*. The peak corresponds to an amplified product from the qPCR run. This melting curve only has one product in all samples and no product in the negative. There are 10 different samples on this curve. Each sample was run in duplicate.

3.6 Statistical Analysis of qPCR Amplification Data

Gene expression analysis for both the *in vitro* propofol and sevoflurane treated mouse brain cortical samples was normalised to the four reference genes. The *t=4 hour* controls were given a fold change of 1 to compare to the treated samples. There was a sample size of *n=5* and the *t=4 hour* treatment was compared to the *t=4 hour* control. However, for both *Bdnf* and *Arc* there was one sample that did not amplify enough to pass the threshold and so the analysis only included a sample size of *n=4*. The $\Delta\Delta C_t$ of qPCR analysis was used (Equation 1) and the data is based on the controls having a regular expression of 1.

3.6.1 Gene Expression Results for Propofol

Following qPCR and statistical analysis, Figure 23 shows that *Bdnf* was downregulated by a fold change of 0.710 and *Arc* was upregulated by a fold change of 1.560. Table 16 contains the p-values generated by a two-tail Student's t-test. *Arc* mRNA transcripts were 11 times more abundant than *Bdnf* mRNA transcripts in the control samples. This was increased to 24 times more *Arc* in the treated sample as shown by Figure 24. Raw data can be found in Appendix 4

Table 16: Gene Expression Results for Propofol. P-value is calculated from a two-tailed Student's t-test.

	Bdnf	***Arc***
Fold Change Average	0.710	1.560
Standard Error	0.147	0.401
P-Value	0.142	0.288

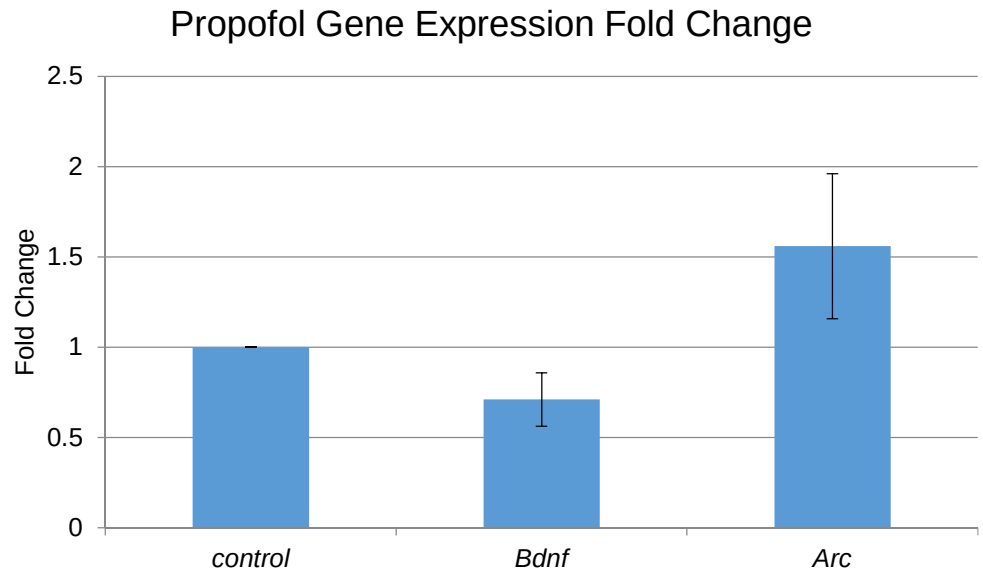


Figure 23: qPCR Gene Expression Analysis of Propofol. *Bdnf* is downregulated while *Arc* is upregulated. The error bars are the standard error for each gene.

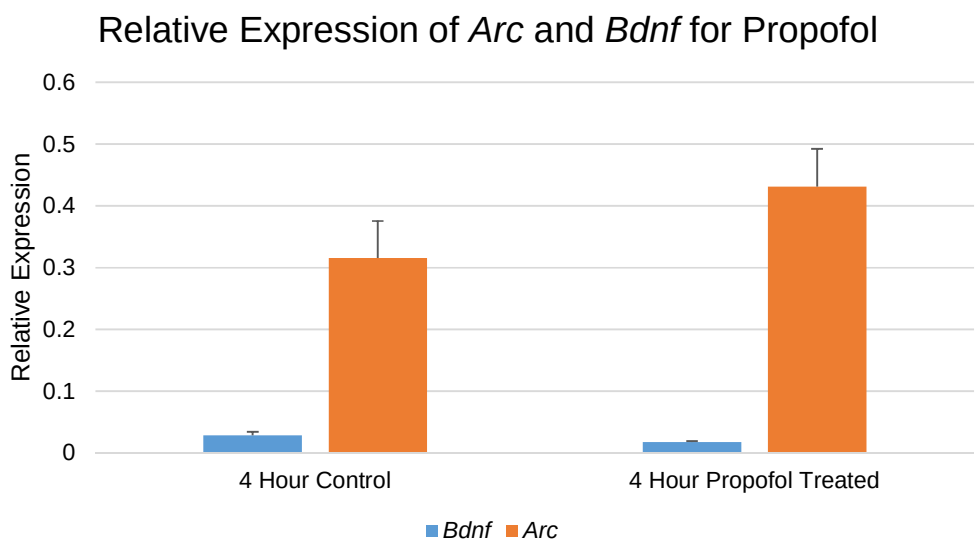


Figure 24: Relative Expression of *Bdnf* and *Arc* for Propofol. *Arc* mRNA transcript quantity within the control samples are roughly ten times the amount compared to *Bdnf*.

3.6.2 Gene Expression Results for Sevoflurane

Figure 25 shows *Bdnf* was upregulated by a fold change of 1.859. Arc was downregulated by a fold change of 0.348 which was statistically significant with a p-value of 0.003. Table 17 contains the p-values generated by a two-tail Student's t-test. *Arc* mRNA transcripts were 37 times more abundant than *Bdnf* mRNA transcripts in the control samples. This was decreased to 9 times more *Arc* in the treated sample as shown by Figure 26. Raw data can be found in Appendix 4.

Table 17: Gene Expression Results for Sevoflurane. P-value is calculated from a two-tailed Student's t-test.

	Bdnf	***Arc***
Fold Change Average	1.859	0.348
Standard Error	0.363	0.034
P-Value	0.139	0.003

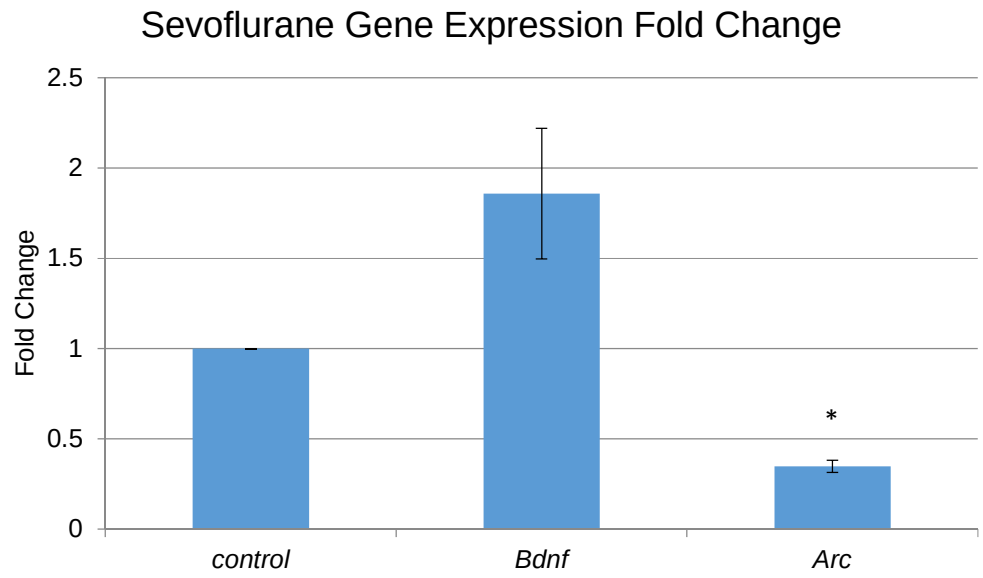


Figure 25: qPCR Gene Expression Analysis of Sevoflurane. *Bdnf* is upregulated while *Arc* is downregulated. * shows statistical significance (p<0.05)

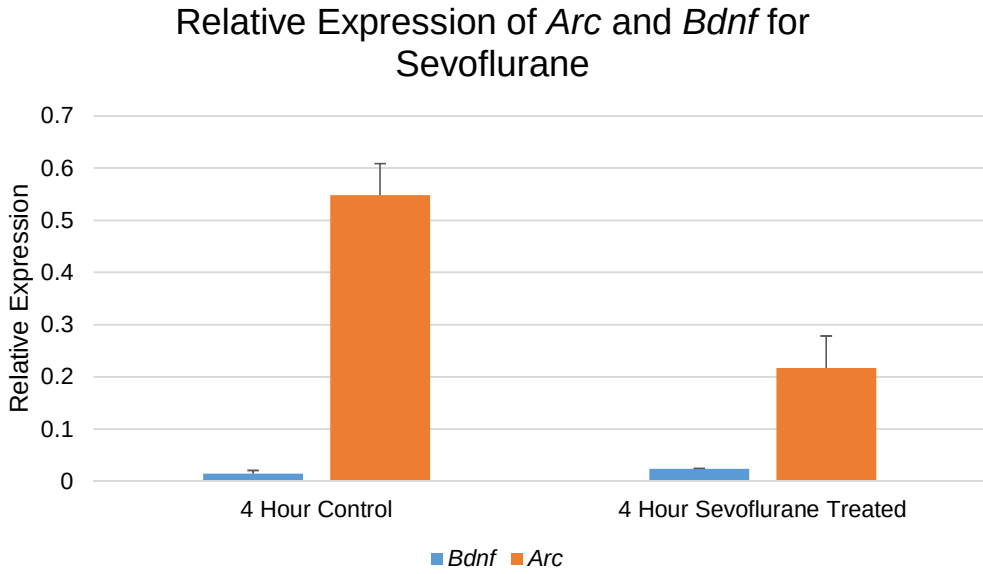


Figure 26: Relative Expression of *Bdnf* and *Arc* for Sevoflurane. As the same with Propofol, *Arc* mRNA transcript quantity within the control samples are roughly ten times the amount compared to *Bdnf*. This changes to be roughly five times the amount in the sevoflurane treated sample.

3.7 Bradford Assay

Bradford assay was carried out to ascertain the protein concentration of the six whole mouse organ extracts. Protein standards were plated out and graphed to produce a standard curve which the absorbance values of the organ extracts were compared against. The standard curve is shown in Figure 27.

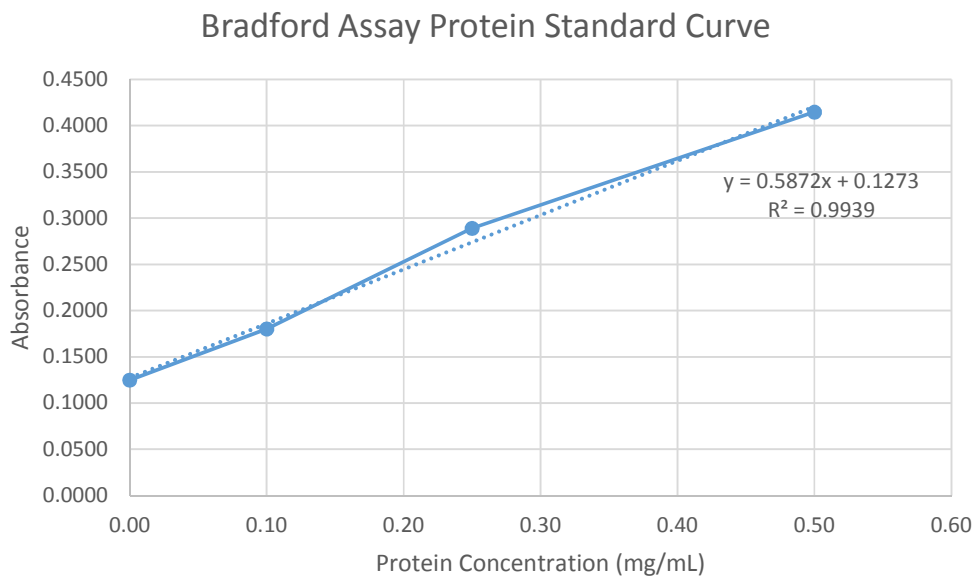


Figure 27: Protein Standard Curve from the Bradford Assay. Protein standards started at a concentration of 0 mg/mL and went to 0.5 mg/mL. The line of best fit gave an equation of y = 0.5872x + 0.1273 with an R-value of 0.9939.

Only up to the 0.5 mg/mL standard was used to produce a line of best fit as past this value the graph started to plateau. Whole organ extracts were diluted by 1:100 to bring the absorbance readings into the accurate readable range. These values and the final protein concentrations are shown in Table 18. A protein range of 4 – 21 mg/mL was extracted. Thus, sufficient concentrations for western blot analysis. Coomassie results indicated protein was not degraded and distinct bands were seen between 10 – 120 kDa (data not shown).

Table 18: Absorbance Values and Concentrations of Mouse Organ Extracts. Large concentrations from the Heart and Lung may have been from left over blood within the tissue.

Organ	Absorbance	Concentration (mg/mL)
Liver	0.1536	4.4789
Spleen	0.1548	4.6747
Kidney	0.1631	6.0967
Brain	0.1780	8.6257
Lung	0.2055	13.3174
Heart	0.2523	21.2790

3.8 Western Blot

Quantitative PCR data suggests that *Bdnf* mRNA levels change after exposure to propofol or sevoflurane. As a change in mRNA quantity doesn't always correlate to a change in protein levels, protein analysis was carried out. Western blots and IHC were carried out concurrently due to the time constraints of this study. Western blots were performed to verify that the Bdnf protein was present within the mouse brain and to validate the commercial antibody that would be used for IHC. The Bdnf antibody N-20 from Santa Cruz Biotech was selected due to the high citation count and positive reviews. The predicted molecular weight of the Bdnf precursor protein is 32 kDa and 14 kDa for the mature protein. However, the western blot produced a band at roughly 55 kDa as shown in Figure 28.

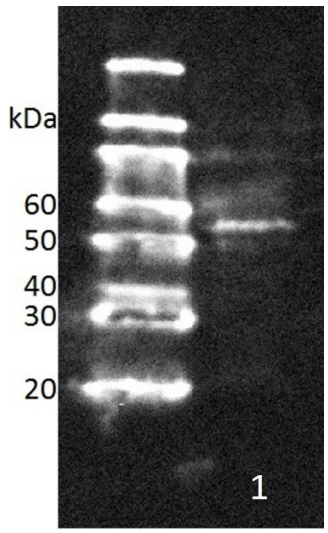


Figure 28: Santa Cruz Biotech Bdnf N-20 Antibody Western Blot on Whole Mouse Brain Extract. 12% SDS-PAGE electrophoresis at 140 V for 50 minutes. 50 µg of protein was loaded. There is one band present at 55 kDa. This band may be an off target binding or a polymer of precursor and mature Bdnf. Genscript WB-MASTER protein standard ladder is included.

As the Bdnf N-20 antibody is a polyclonal antibody, the band could have come from either off target binding or polymer combinations of the precursor and mature protein. A Beta-actin positive control was run on brain extract at three different concentrations to ensure the protocol was working correctly. A band was seen at 42 kDa, which is the correct predicted size. This is shown in Figure 29.

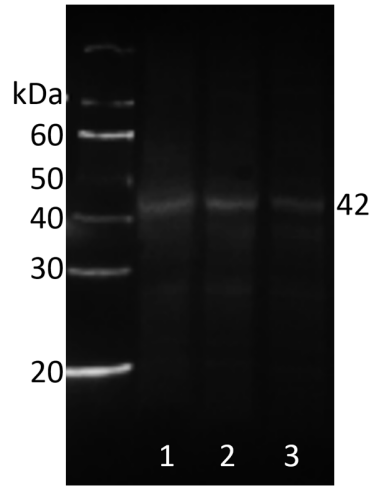


Figure 29: Genetex Beta-actin GTX110564 Antibody Western Blot on Whole Mouse Brain Extract. 12% SDS-PAGE electrophoresis at 140 V for 50 minutes. 1) 50 µg protein loaded. 2) 12.5 µg protein loaded. 3) 5 µg protein loaded. Genscript WB-MASTER protein standard ladder is included.

The Laemmli buffer used to denature and reduced the sample during the boiling step only had a 10% β-mercaptoethanol concentration so to rule out the formation of protein polymers this concentration was increased to 25%. The primary antibody concentration was increased from 1:1,000 to 1:100 and a protein concentration gradient was also included to ensure the protein concentration was high enough to show the 14 kDa and 32 kDa proteins. The western blot showed a band at 55 kDa again with no bands at 14 kDa or 32 kDa as shown in Figure 30.

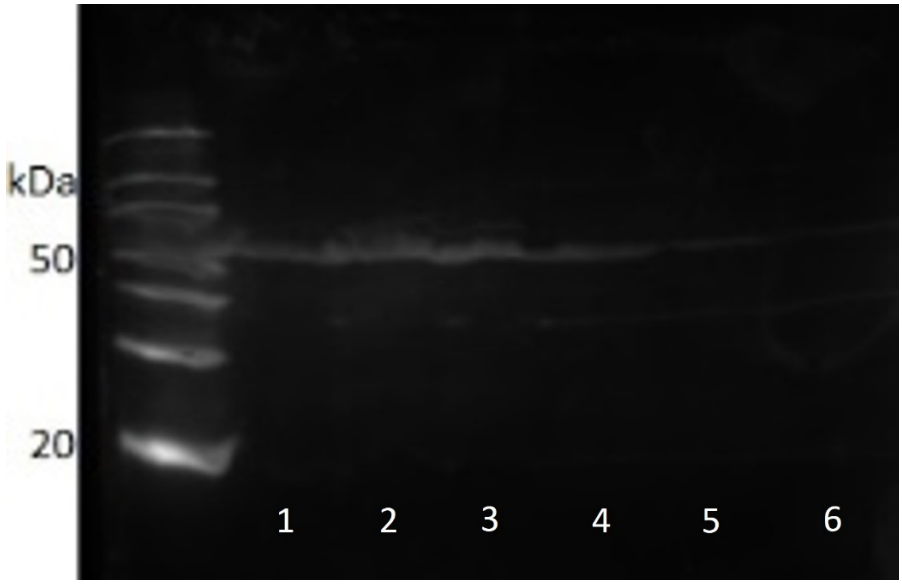


Figure 30: Santa Cruz Biotech Bdnf N-20 Western Blot with Higher Protein Concentration of Mouse Whole Brain Extract. 12% SDS-PAGE electrophoresis at 140 V for 50 minutes. Protein loading quantity: 1) 100 µg 2) 50 µg 3) 25 µg 4) 10 µg 5) 5 µg 6) 2 µg. There is only one band at 55 kDa.

The conclusion from this set of western blots was that the Santa Cruz Bdnf N-20 antibody we received was defective so the Abcam ab72439 Bdnf antibody was ordered. The predicted size of the ab72439 antibody is 28 kDa. This western blot was run with whole extract from mouse brain, kidney and heart. A band was observed at 28 kDa as shown in Figure 31. Extra bands were observed at 52 kDa in the kidney sample which could be protein polymers or off target binding to other neurotrophic factors. This western blot was replicated with the same result as Figure 31 (data not shown). Poceau S stain shown in Figure 32.

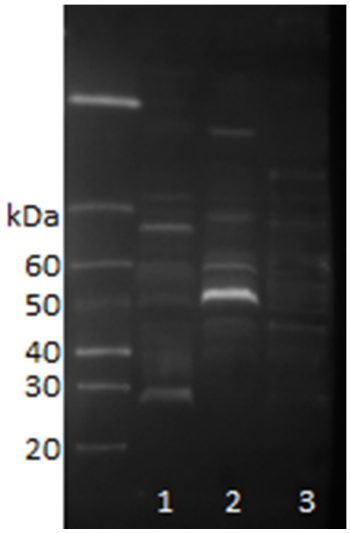


Figure 31: Abcam Bdnf ab72439 Antibody Western Blot. 4%-16% SDS-PAGE electrophoresis at 150 V for 50 minutes. 1) Mouse whole brain extract. 2) Mouse whole kidney extract. 3) Mouse whole heart extract. Each extract had a protein concentration of 50 µg of sample was loaded into each well.

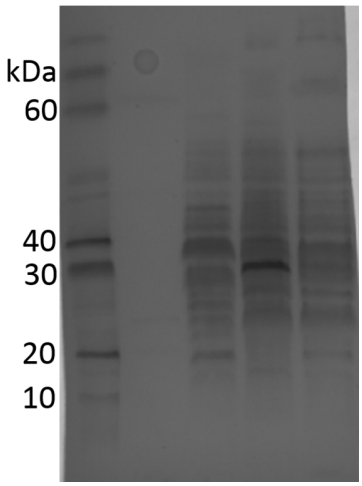


Figure 32: Ponceau S Stain of Membrane. The three lanes show protein transfer onto the membrane was successful. PAGE-MASTER GenScript ladder is included.

In the brain extract there is a band at 28 kDa as predicted but a range of high molecular weight bands including a band at 68 kDa which could be a polymer or off target binding. There is a group of bands at 50 kDa in the kidney extract which could be protein polymers and a slight band at 28 kDa in the heart extract. Bdnf is not in as high protein concentration in the heart as in the brain.

3.9 Hematoxylin and Eosin Staining

H&E was carried out to ensure the cells of the IHC sections were still viable for IHC and to have a reference to navigate the IHC sections. The neocortex is dense in nuclei which is shown by the violet colour from the hematoxylin stain in Figure 33.

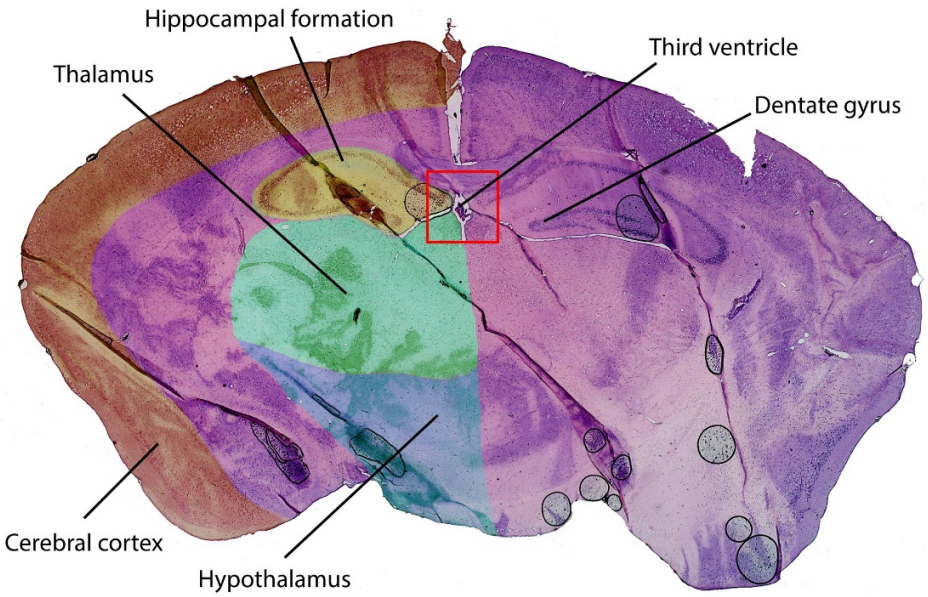


Figure 33: H&E Staining of a Mouse Brain Coronal Cryosection. Tissue is from a female mouse aged 10 weeks and section in OCT. The cells of the section are still viable as shown by the hematoxylin staining the nuclei of the neocortex cells. Air bubbles are present from the coverslip procedure. The third ventricle (red box) is the location that most IHC pictures were taken with the microscope camera. Pictures were also taken at the dentate gyrus.

3.10 Immunohistochemistry

IHC was carried out to validate the presence of the Bdnf protein within the neocortex and to compare Bdnf protein levels between brain slices that were treated and untreated with propofol for *t=4 hours*.

IHC with the Santa Cruz Bdnf N-20 antibody produced sections that had a uniform green fluorescence that was also observed at the similar levels in the negative control sections as shown in Figure 34. This tissue was fixed in 4% PFA overnight at 4 °C prior to being frozen in OCT for sectioning to preserve the tissue.

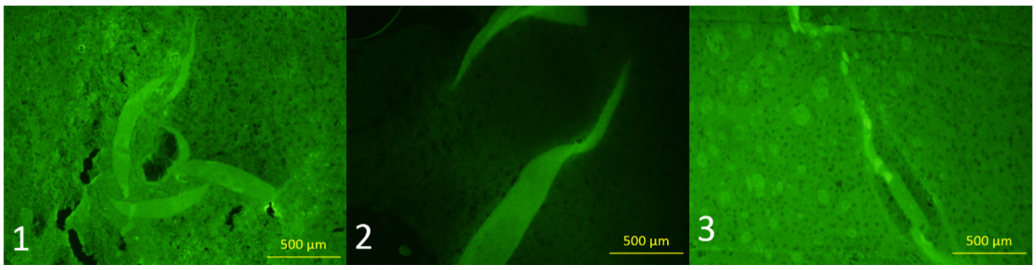


Figure 34: IHC Microscopy Section of an Untreated Mouse Brain with N-20 Antibody. FITC secondary antibody at 40X magnification. 1) Third ventricle 1:1000 secondary antibody concentration. 2) Dentate gyrus 1:500 secondary antibody concentration. 3) Dentate gyrus negative control containing no primary antibody. The background is high in these sections with the negative control having just as high background. Fixing of the tissue before sectioning could have been the cause.

Fixation of the brain tissue before being frozen in OCT was removed to reduce the background noise. After the brain tissue was removed from the euthanised mouse it was placed directly into the cryostat mold, covered with OCT liquid and snap frozen. Removing the tissue fixing step reduced the background noise as shown in Figure 35.

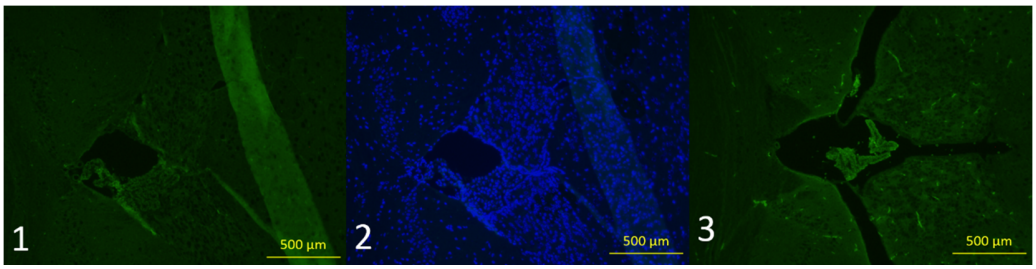


Figure 35: IHC Microscopy Sections of Untreated Mouse Brain with N-20 Antibody, No Tissue Fixation, 30 Minute Section Fixation. Alexa Fluor® 488 secondary antibody at 40X magnification. 1) Third ventricle 1:2000 secondary antibody concentration. 2) Section 1 overlaid with DAPI stain. 3) Third ventricle negative control. Background is lower compared to the sections that were fixed before going into OCT. Negative control has formaldehyde fluorescence around the debris in the middle of the third ventricle.

Based on the western blot analysis for the Santa Cruz Bdnf N-20 antibody it was decided to switch antibodies and use the Abcam ab72439 antibody for IHC.

The section fixation step was reduced from 30 minutes to 10 minutes in 4% PFA. Antigen retrieval was carried out for three minutes in a pH 6.0 citric acid buffer as advised by Abcam. However, the Bdnf antibody did not bind to target protein as shown in Figure 36.

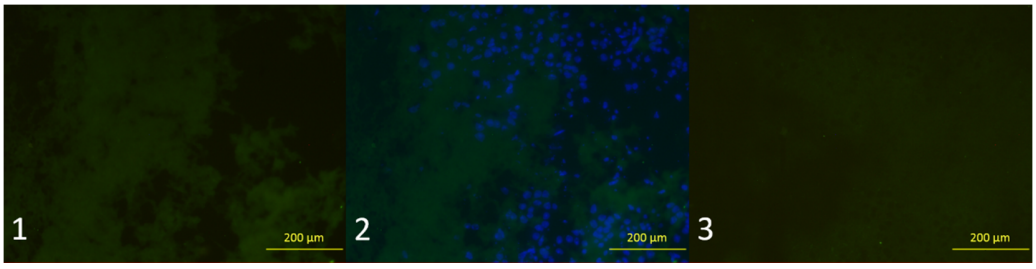


Figure 36: Microscopy Sections of Mouse Brain with Abcam ab72439 Antibody and 10 Minute Fixation Time. Alexa Fluor® 488 secondary antibody at 100X magnification. 1) Third ventricle 1:2000 secondary antibody concentration. 2) Section 1 overlaid with DAPI stain. 3) Dentate gyrus negative control. No discernible binding of the secondary antibody is demonstrated.

Background noise had been further reduced by reducing the section fixation time. The three minute antigen retrieval may not have been long enough to unmask the epitope so this step was increased to ten minutes boiling in pH 6.0 citric acid buffer. Polylysine coated slides were used instead of gelatin coated slides in an attempt to remove background noise. The results are shown in Figure 37.

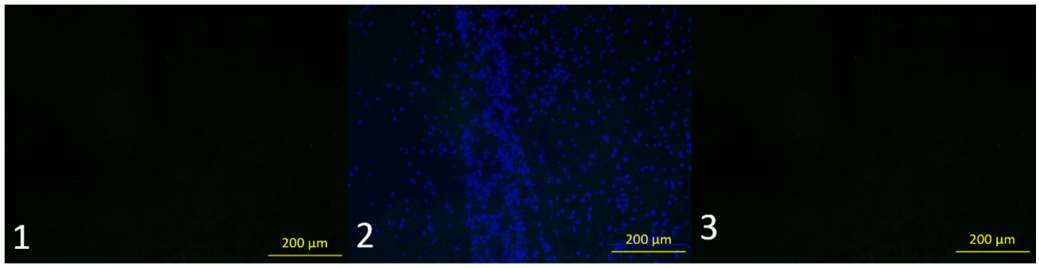


Figure 37: Microscopy Sections of Mouse Brain with Abcam ab72439 Antibody, 10 Minute Fixation Time and Commercial Polylysine Slides. Alexa Fluor® 488 secondary antibody at 100X magnification. 1) Third ventricle 1:2000 secondary antibody concentration. 2) Section 1 overlaid with DAPI stain. 3) Third ventricle negative control. No discernible binding of the secondary antibody is demonstrated.

Background noise had been completely eliminated, however there was no binding of the secondary antibody.

Chapter Four

Discussion

4.1 Experimental Set Up

Experimental conditions after anaesthetic exposure were kept identical to the *in vivo* study (Laura Bell, UoW, NZ; 2014) to reduce variation between *in vivo* and *in vitro* brain samples. Animals were sacrificed at similar times and the brains were placed into ice-cold nACSF to minimise environmental shock while being transported to the Cortical Research Laboratory. Four hundred micrometre thick coronal sections were used to allow the anaesthetics to completely perfuse through the *in vitro* tissue slice. Brain slices were kept in Mg-deficient ACSF to induce seizure-like activity. This electrical activity in the brain slice was measured to ensure each slice was still viable, and able to continue to carry out cell functions, e.g. gene expression, while determining if the anaesthetic exposure was having the expected inhibitory effect on SLE activity. All SLE activity was blocked within 2 hours of anaesthetic exposure and a pilot experiment was conducted (Logan Voss, UoW, NZ; 2015) which tested that SLE activity returned after a 4 hour exposure to anaesthetics to show that the decline in SLE activity was not due to tissue death but due to the anaesthetics. Our criteria for a viable slice was that at least four of the six recorded locations showed SLE activity. This criteria was not based on empirical evidence for viable tissue, but on the fact that having four out of six locations active meant that the majority of the tissue was viable. We attempted to take slices only from the posterior side of the brain to reduce gene expression differences due to different areas of the brain. However if a posterior slice had insufficient activity then slices were taken from the anterior section of the brain. The time spent in the Mg-deficient ACSF was kept constant between all slices to reduce expression differences related to stress on the

brain slices. Carbogen was used to oxygenate and stabilise the pH of the ACSF. During the propofol exposure period, nACSF was fed into the bath enclosure containing the brain slice through a gravity fed drip system which had a constant flow rate between all slices of 5 mL/min. Sevoflurane exposure was accomplished through a syringe pump system which pumped at a flow rate of 5 mL/min. A sample size of *n=5* animals was chosen because of time constraints but also to attempt to gather enough data to have statistically significant results. From each animal a *t=0 hour* control, *t=4 hour* control and *t=4 hour* treatment slice was taken. Only the treatment slice was exposed to propofol or sevoflurane. A post hoc sample size calculation was carried out using G Power software package (Faul, Erdfelder, Lang and Buchner, 2007) which showed that a sample size of *n=25* animals for each anaesthetic would be required to form statistically accurate data for all changes in expression [81].

4.1.1 Propofol Experiment

Propofol was mixed with intralipid, the vehicle used for clinical intravenous use, and diluted into ACSF to a final concentration of 10 µg/mL. This concentration of propofol was higher than clinically relevant intravenous levels due to propofol diffusing slowly through brain tissue that is devoid of a blood supply. However, this produced propofol concentrations within the brain slices corresponding to those achieved *in vivo* [34]. The flow rate was kept constant between slices to ensure similar concentrations of propofol within the tissue.

4.1.2 Sevoflurane Experiment

To deliver the vapour anaesthetic sevoflurane it was added to 50 mL of nACSF in a syringe and pumped into the bath containing the brain slice. The level of sevoflurane in the ACSF was at a clinically relevant concentration of 1 MAC as shown by previous studies [5, 11]. A vapouriser could be used to allow for a more accurate concentration of sevoflurane and simpler delivery; however, one could not be sourced for this experiment.

4.2 RNA Quantification and Qualification

RNA qualification and quantification was carried out to ensure good quality RNA, as low quality RNA would compromise results from quantitative analysis including qPCR analysis, and equal loading of RNA template in downstream applications. The NanoDrop™ 2000 Spectrophotometer was used to assess quality and quantity of 30 RNA samples and agarose gel electrophoresis was also used to determine that the 10 *t=0 hour* control RNA samples were not degraded.

4.2.1 Spectrophotometer Analysis

Spectrophotometer analysis can determine the quantity and purity of DNA and RNA within a sample. This is accomplished by absorbance ratios of the 260 nm and 280 nm wavelengths which compares the quantity of RNA or DNA in a sample to contaminants such as proteins. The target absorbance ratio is between 1.8 and 2.0 which qualifies the RNA sample to be considered pure or lacking contaminants. Sample P5T4C had a low absorbance ratio at 1.73. This is most likely because of phenol carryover from the TRI Reagent® extraction process. A second 75% ethanol wash step could be added to the end of the RNA extraction protocol to further reduce the phenol carryover and consistently get high A_{260}/A_{280} ratios. However, this was not done as the P5T4C sample was the only sample that had an absorbance ratio outside of the target range. S5T0C had a low RNA concentration at 164.1 ng/µL. This would have most likely been from not successfully separating all of the aqueous layer which contained the RNA during the extraction protocol or excessive drying of the RNA pellet; therefore, receiving less RNA template.

In place of the NanoDrop 2000, a bioanalyser could be used to achieve more accurate readings of RNA quantity and quality. A bioanalyser is considered more accurate due to the RNA integrity number (RIN) algorithm which takes into account the complete RNA spectrum and not just the 18S and 28S rRNA

regions [82]. A RIN is produced ranging from 1 to 10 where 1 is the most degraded on this scale while 10 is the most intact. Also, using computer software to qualify the integrity of an RNA sample removes human error that is associated with gel electrophoresis data.

4.2.2 Non-Denaturing Gel Electrophoresis

Agarose gel electrophoresis was carried out to assess the quality of the RNA template. All samples were not run because there was not enough RNA sample to analyse on an agarose gel and complete the qPCR analysis. Therefore, the zero hour controls were used to infer the quality of the samples used for qPCR analysis. Non-denaturing gel electrophoresis was used instead of denaturing formaldehyde gel electrophoresis because the stock of formaldehyde and MOPS buffer was of poor quality (Linda Peters, personal communication, April 9th, 2015). Inclusion on an RNA molecular weight ladder on a denaturing gel will resolve the RNA as single stranded ribosomal bands, preventing the formation of any secondary structures. Thus, the 18S and 28S will migrate at the true size of 1869 bp and 4712 bp respectively, where the 28S band is roughly twice as bright as the 18S band. This demonstrates that the RNA is intact and has not degraded. An alternative, cheaper and less time-consuming, method was to use standard non-denaturing TAE gels to examine if two sharp bands representing the 18S and 28S ribosomal bands were present. The first RNA sample extracted did not show the two expected bands but instead a smear on the gel. The DEPC-treated water used to resuspend the RNA after extraction was replaced and all subsequent RNA samples showed no smearing on the gel. Both the propofol and sevoflurane *t=0 hour* control RNA samples were run on gels and the two rRNA bands were seen. Thus, data suggests the RNA was intact and suitable for downstream application.

In place of the denaturing formaldehyde gel electrophoresis, bleach gel electrophoresis could have been carried out. This involves the use of a TAE agarose gel containing 0.5 – 1% bleach and has been shown to produce bright,

sharp bands with 1 µg of RNA. Bleach has been postulated to denature the secondary structures of RNA and destroy any RNases that are present within the electrophoresis setup and reagents. Bleach gel electrophoresis allows the rapid analysis of RNA quality without the use of toxic chemicals used in denaturing formaldehyde gels [83].

Alternatively, a new molecular method published this year using differential amplicons (ΔAmp) to assess RNA degradation could be used. This measures ΔAmp of a reference gene and an Endogenous RNase Resistant (ERR) within an RNA sample. The degree of RNA damage is reflected by the difference of the differential expression of the reference gene to the ERR marker. This method is more powerful for analysing RNA degradation compared to the bioanalyser as it assesses the mRNA damage where the bioanalyser is dominated by rRNA [84].

4.2.3 Genomic DNA Contamination

Before cDNA synthesis, each sample subjected to a DNAse treatment protocol due to TRI Reagent® RNA extraction not removing all genomic DNA. Standard PCRs were run with the cDNA –RT controls to confirm all genomic DNA had been removed from the sample. Genomic DNA contamination persisted after the DNAse treatment so the protocol was changed and the 37 °C incubation step was increased from 15 minutes to one hour. This increase in DNAse treatment time consistently removed all genomic DNA from the RNA samples. A non-exon boundary primer was used to assess the level of genomic DNA in the -RT control. This primer was for the housekeeping gene *Gapdh*. *Gapdh* and *B2m* housekeeping gene primers used to verify the ability to amplify up the cDNA template if successful cDNA synthesis had occurred. *Bdnf* primer was included to see whether or not *Bdnf* could be amplified; however, this was not used as an indication of the presence of *Bdnf*. If there was no amplification of *Bdnf* with standard PCR the sample was still used in qPCR analysis because of its higher sensitivity.

4.3 Quantitative PCR Analysis

Quantitative PCR was used to measure the relative expression of our genes of interest, *Bdnf* and *Arc*. These two genes were chosen based on data published (Laura Bell, UoW, NZ; 2014) regarding a study that showed that *Bdnf* and *Arc* were differentially expressed after anaesthetic exposure to a mouse brain *in vivo.* Also, based on the role these two genes have in memory consolidation [22, 45]. The *Bdnf* and *Arc* primers used in this study were previously sequenced by Laura Bell. RNA was extracted from only 12 mg of tissue, which made template cDNA sample volume an issue. This forced us to dilute the cDNA samples to a concentration of 1:5. This provided enough sample volume for all qPCR runs and a buffer for qPCR runs that had to be repeated.

No-template controls were run to verify there was no contamination of the PCR reaction. Contamination did occur, particularly in the *Actb* runs. This may have been because *Actb* is a commonly used primer in the Laboratory of Molecular Genetics and so *Actb* product is spread throughout the lab as an aerosol. Steps were taken to prevent contamination which included use of a PCR hood, not wearing a lab coat, which could have harboured contaminating amplicons, and using DEPC-treated mQH_2O. If contamination occurred then the run was repeated, but with primer solutions being remade. Non-amplification occurred in one replicate of a sample during two different qPCR runs, which was most likely from human error during pipetting of the cDNA template. When this occurred, the run was also repeated.

According to the MIQE guidelines at least 2 reference genes are needed to normalise qPCR data [68]. A reference gene must be expressed equally throughout different pathophysiological conditions to be used in qPCR normalisation. *Gapdh*, *β2m*, *Actβ* and *HPRT1* reference gene primers were used for qPCR normalisation. These genes were chosen because they were confirmed to be reference genes in previous studies [69, 79, 80].

The qPCR assay conditions were validated by Keiran Oxton (UoW, NZ; 2013) and Laura Bell (UoW, NZ; 2014). A melt at 80 °C was included in every qPCR cycle before fluorescent acquisition to remove primer dimers which gives a more accurate quantification.

4.3.1 Melt Curves

Melt curves were included at the end of a qPCR run to assess the product or products formed during the qPCR run. The melt graph was formed using the negative first derivative of the melting curve from the melt cycle. The negative first derivative was graphed to give a clearer indication of where the melting point is located. The melting point is the temperature at which 50% of the dsDNA has been dissociated. A melting point of a product is determined from the length of the sequence and the GC content of the sequence. As G-C bonds contain three hydrogen bonds compared to the two hydrogen bonds of an A-T bond, a sequence with a higher GC content will have a higher melting point. Because of this we are able to determine if two products differ more accurately than through gel electrophoresis.

If a NTC amplified up above baseline then the product melting point was examined to determine if the product was the same as the one present in the samples. If the products differed then the sample was not repeated and the data was used in the statistical analysis.

4.3.2 Statistical Analysis

The $\Delta\Delta C_t$ method of qPCR analysis was used. This method uses C_t values which are cycle numbers based off of a threshold set on the computer. The geometric mean of all the control gene C_t values are taken away from the C_t values of the genes of interest for both the control samples and the treatment samples to give a ΔC_t value. The ΔC_t of the control samples are taken from the ΔC_t value of the treatment samples to give a $\Delta\Delta C_t$ value and the fold change difference for a gene of interest is $2^{(-\Delta\Delta Ct)}$ [85]. Equation 1 provided the formula for the $\Delta\Delta C_t$ method.

Each reference gene was subsequently removed from the geometric mean and then replaced to ensure that one reference gene was not skewing the data. If one of the reference genes skewed the data then this would indicate that that particular reference gene changed due to the different pathophysiological conditions between the control and treatment samples and would not be suitable as a reference gene [86].

The different relative expression levels of both *Bdnf* and *Arc* varied greatly between samples within the propofol and sevoflurane experiments. With a sample size of *n=5* a fairly accurate average was seen; however, there were some outliers that showed the opposite change in gene expression compared to the overall average. This could be due to the differences in expression profiles between the posterior and anterior side of the neocortex. We aimed to take slices from the same area of the brain for each sample; however, this was not always possible if there were no viable slices from the posterior side of the brain. Another explanation is that a difference in SLE activity or subtle variations in tissue health, which was not assessed by our viability criteria, could have affected expression levels within the brain. Having a higher SLE activity may have amplified the effect that a general anaesthetic had on the brain slice.

4.3.3 *In vitro* Gene Expression Analysis of Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor is a protein in the neurotrophin family which is important in neuron survival and encouraging the formation of new neurons and synapses [42, 43]. It has been shown that the transcription of *Bdnf* is required for maintenance of late-phase LTP and so may be necessary for memory consolidation and recall [50]. Lowered *Bdnf* levels has been linked to neurodegenerative diseases including Alzheimer's disease, Huntington's disease, Parkinson's disease and dementia, but also mental illnesses such as schizophrenia, depression and epilepsy [87, 88, 89, 90].

Our gene expression analysis on *Bdnf* after a *t=4 hour* exposure of propofol shows a downregulation of the gene by factor of 0.71; however, this was not statistically significant. Previous studies has shown that *Bdnf* mRNA levels are decreased in rat hippocampal neurons from GABAergic stimulation after neuronal maturation. Due to propofols proposed mechanism of action of GABA enhancement, it is possible that this is one of the causes of reduction in *Bdnf* levels [91]. It has also been shown that *Bdnf* mRNA is down regulated upon activation of the 5-HT receptors (serotonin receptors); therefore, general anaesthetics could increase the level of serotonin within the brain which activate these 5-HT receptors, thereby reducing *Bdnf* expression [92, 93, 94].

Bdnf directly activates NMDA receptor subunit one through phosphorylation and NMDA receptor subunit 2-β through the Fyn signaling pathway [95, 96]. Activation of NMDA receptors causes molecular changes that are required in the formation of new memories. A reduction in *Bdnf* levels would decrease the activation of NMDA receptors and, therefore, impede the formation of new memories [97]. This can explain the loss of memory consolidation both during and after surgeries where propofol is used. An increase in NMDA receptor activity can form new memories but the AMPA receptors maintain the synapses communication, structure and function long after the initial activation of the NMDA receptors and channels. *Bdnf* has been shown to upregulate the AMPA receptors, GluR1 and GluR2 through the TrkB pathway [98, 99]. A reduction in *Bdnf* levels would decrease the number of AMPA receptors and, therefore, reduce the likelihood that a new memory is consolidated to long term memory. This would explain the amnesic effect that propofol has on patients before it is administered.

We observed an upregulation of *Bdnf* after *t=4 hour* exposure of sevoflurane by a factor of 1.86; however, this was not statistically significant. *Bdnf* has been shown to be upregulated in the hippocampus of epileptic patients after a

seizure [100]. An increase in *Bdnf* during the anaesthetic exposure time could be due to the seizure-like activity that is induced within the tissue to ensure the tissue was still viable. However, the propofol samples had a decrease in *Bdnf* expression but it may be that the net decrease of *Bdnf* expression due to GABA enhancement and activation of the 5-HT receptors is greater for propofol than for sevoflurane.

4.3.4 *In vitro* Gene Expression Analysis of Activity-Regulated Cytoskeleton-Associated Protein

Activity-regulated cytoskeleton-associated protein is a protein from the immediate-early gene family. *Arc* is expressed after a learning experience and rapidly transported to dendrites. *Arc* plays a role in neuronal plasticity through the endocytosis of AMPA receptors, regulation of cell morphology, cytoskeletal organisation and modulating dendritic spine remodeling and density [52]. *Arc* has been implicated in neurological conditions such as amnesia, Alzheimer's disease, and autism spectrum disorders [101, 102].

Our gene expression analysis on *Arc* after a *t=4 hour* exposure of propofol showed an upregulation of the gene by factor of 1.56; however, this was not statistically significant. *Arc* is regulated by many different pathways including *Bdnf* [52, 103]. As we see *Bdnf* is down regulated in the propofol experiments, we might expect *Arc* to also be down regulated; however, this is not the case. *Arc* being up regulated could be due to its ability to be managed by proteins and pathways outside of the neurotrophic family of proteins. These proteins include epidermal growth factor and nerve growth factor but also the glutamate binding to NMDA receptors and dopamine binding to dopamine receptor D_1 [104, 105, 106]. Therefore, a reduction in *Bdnf* levels could be rectified by activating a wide range of other pathways to save *Arc* levels.

We observed a downregulation of *Arc* after *t=4 hour* exposure of sevoflurane. This downregulation was by a factor of 0.348 and was statistically significant with a p-value of 0.003. This data agrees with previously published data by

Kobayashi et al. (2007) who also noted a downregulation of *Arc* in response to sevoflurane induced anaesthesia. *Arc* is expressed after activity at the synapse, and when this activity is reduced due to general anaesthesia, it is expected that there will be a reduction in *Arc* mRNA levels [54]. Actin is a protein which forms microfilaments. In its filamentous form (F-actin), it is used for cell signaling and remodeling of cells [107]. As synaptic plasticity requires NMDA and AMPA receptors to be moved to synaptic sites, the F-actin molecules are used for this protein movement within the synapses. A decrease in *Arc* levels may inhibit the formation of F-actin and, therefore, stop synapses from being remodeled for synaptic strengthening. This would stop the consolidation of short term memory into long term memory. *Arc* also modulates dendritic spine density which creates more synaptic sites for memory consolidation [108]. Reducing *Arc* levels may possibly stop the formation of new synaptic sites and therefore stop the formation of new memories.

4.3.5 Gene Expression Differences between *In vitro* and *In vivo*

Data from both the *t=4 hour* propofol *in vivo* exposure study conducted by Laura Bell (UoW, NZ; 2014) and our *in vitro* study showed a slight downregulation of *Bdnf,* but neither result was statistically significant. In the *t=4 hour* sevoflurane *in vivo* exposure study showed a statistically significant decrease in *Bdnf* mRNA levels while our *t=4 hour* sevoflurane *in vitro* found an increase in *Bdnf* mRNA levels. *Arc* mRNA levels after a *t=4 hour in vivo* propofol treatment showed a statistically significant increase. Our experiment also found an increase in *Arc* mRNA levels after a *t=4 hour* propofol *in vitro* exposure. No change in *Arc* mRNA levels after a *t=4 hour in vivo* sevoflurane treatment were seen, while after a *t=4 hour* sevoflurane *in vitro* exposure a statistically significant decrease in *Arc* levels was seen. These results are shown in Table 19.

Table 19: Gene Expression Difference between *In vitro* and *In vivo*. ↑ signifies upregulation. ↓ signifies downregulation. – signifies no change. Only the results marked by a * were shown to be statistically significant (p<0.05).

	In vitro		***In vivo***	
	Bdnf	**Arc**	**Bdnf**	**Arc**
Propofol	↓	↑	↓	↑*
Sevoflurane	↑	↓*	↓	-

Bdnf has been shown to be up regulated in epileptic patients due to seizures [100]. As our *in vitro* experiment required the induction of seizure-like events in the brain slices to verify the validity of the tissue, these seizures may have been the cause of increase *Bdnf* levels in the *in vitro* experiment. For the *in vivo* experiments the whole neocortex was collected for RNA extraction while the *in vitro* experiment used slices to ensure complete perfusion of the general anaesthetics. We saw a difference between individual slices from the *in vitro* experiments and, recently, single cell transcriptomic work has shown that cells in close proximity can have different transcriptomes. Therefore, the differences we see between the *in vivo* and *in vitro* work could be due to the fact that the *in vivo* experiment effectively took an average transcriptome of the whole brain while we took the transcriptome of the posterior side of the brain [109, 110].

4.4 Detection of Protein Expression

IHC was performed for detecting protein level localisation within the neocortex, and western blots to validate the primary antibodies that were going to be used on whole tissue mouse brain extracts. The Santa Cruz N-20 Bdnf polyclonal antibody was selected based on its high citation record in the literature. We expected to observe a band at either 14 kDa or 32 kDa. However, we observed a 55 kDa band on our western blot using this antibody against epitope sample of whole brain mouse lysate. The larger band may have been from off-target binding due to the antibody being polyclonal or protein polymer formation.

Off-target binding would be to other proteins within the neurotrophin family which includes; Nerve Growth Factor (NGF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4). All of these neurotrophins have similar protein structures. To attempt to improve antibody recognition as intended, we first increased the β-mercaptoethanol concentration in the sample buffer in order to help break bonds between potential protein polymers. This did not change the western blot and a band at 55 kDa was still produced with no sign of a band at the predicted sizes. An increase in protein concentration (50 µg to 100 µg) was used to remove the possibility of the predicted band sizes being lost due to low protein concentrations. Whole tissue extracts from five other mouse organs, which included; heart, lung, kidney, spleen and liver, were also run on a western blot to remove the possibility of the antibody binding to a Bdnf protein that has different protein splicing or post-translational modifications not present within the mouse brain. No bands at the predicted sizes were seen but an increasing intensity of the 55 kDa band was observed as protein concentration was increased. A western blot was run with the Genetex GTX110564 Beta-actin antibody which produced a band at the correct size of 42 kDa indicating that the western blot procedure was not flawed. Also a non-denaturing PAGE gel was used to ensure that the denaturing gel was not removing the epitope that the Bdnf antibody recognised (data not shown). The same result was produced with a single band at 55 kDa. Lastly, an increase in primary antibody concentration, from 1:1000 to 1:100, was used which produced the same results as increasing the protein concentration. Protein transfer from the SDS-PAGE gel to the PVDF membrane occurred correctly as evident from the Ponceau S staining. In conclusion, this antibody did not recognise its expected epitope and generate bands of the expected size. It was noted that this antibody was not stored correctly upon arrival and thus, the stability of the antibody may have not been optimal (Linda Peters, personal communication, January 15th, 2015). Therefore, a new antibody, Abcam ab72439, was ordered.

The Abcam polyclonal ab72439 antibody produced western blots with the expected size of 28 kDa. There were other bands on this gel which could be attributed to a high secondary antibody concentration. Decreasing the secondary antibody concentration or increasing the final wash step duration would remove these extra bands. The positive result was replicated and the antibody was validated. The Abcam antibody was then used in IHC to determine areas and levels of Bdnf expression within the mouse brain.

4.5 Hematoxylin and Eosin Staining

H&E staining was used to detect changes in cell morphology within the mouse brain between a control section and a treated section. It was also used to counter stain IHC sections to enable us to determine the cell type we were viewing under fluorescence. Ten micrometre thick mouse brain coronal cryosections were sectioned in a cryostat from a whole mouse brain and placed onto commercial polylysine slides. H&E staining procedure was used to examine the histological appearance of the cells. The H&E stained sections showed the anatomy of the mouse brain coronal section clearly. The nuclei rich areas of the neocortex and hippocampal formation were stained blue by the hematoxylin. This indicated that the cryosection procedure produced sections with intact cells.

There was folding and tearing of the tissue during the cryosectioning procedure which meant that no clear and complete sections were produced. Molds containing the brain tissue in OCT were snap frozen and placed into a -80 °C freezer to ensure the tissue was completely frozen. A new blade was used and sectioning at -30 °C and – 15 °C was attempted with no improvement to the folding and tearing of the tissue. Also, sectioning at a thickness of 5 µm instead of 10 µm did not improve the section quality. Tissue from multiple different mice were used with no difference in the quality of sections. Further optimisation of the cryosectioning procedure is needed to improve sections.

4.6 Immunohistochemistry

IHC was carried out to validate the presence of the Bdnf protein within the neocortex, and to compare Bdnf protein levels between brain slices that were treated and untreated with propofol for *t=4 hours*. All of the IHC pictures were taken at the center of the *corpus callosum,* including part of the third ventricle for comparison.

No fluorescence above baseline, which was established from control slides that contained no primary antibody, was identified in any of the microscope sections. However, the DAPI stain worked correctly and stained the nuclei bright blue. A wide range of problems were encountered during IHC and Table 20 outlines the troubleshooting methodologies used to try an overcome these issues

Table 20: Troubleshooting IHC Problems.

Sections tearing and folding	Cutting temperature trailed at -15 °C, -25 °C and -30 °C
High background	Removed tissue fixation in 4% PFA before cryosectioning.
	IHC protocol fixation reduced from 30 minutes to 10 minutes.
	Blocking solution was changed from 5% goat serum to Lifetech Blockaid.
	Changed from handmade gelatin-coated slides to commercial polylysine coated slides.
N-20 antibody not binding to Bdnf	Primary antibody concentration gradient from 1:1000 to 1:100. Secondary antibody kept at 1:2000 as advised by the manufacturer's data sheet.
	Overnight incubation of slides in primary antibody at 4 °C.
Ab72439 antibody not binding to Bdnf	Increased antigen retrieval boiling time.
	Increased primary antibody concentration from 1:100 to 1:50. Secondary antibody kept at 1:2000.

We successfully removed all background with reducing the fixation times and changing from handmade gelatin coated slides to commercial polylysine coated slides. However, we were unable to detect a positive green Bdnf signal within the brain tissue sections. Reintroducing the dehydration step that was removed after tissue was placed directly into OCT would cause the tissue to be more stable and perhaps not tear as much. Further increasing antigen retrieval time could allow the Bdnf antibody to bind to the protein on IHC sections.

Chapter Five

Conclusion

The aim of this study was to identify changes in the expression, within the neocortex, of the memory-related genes, brain-derived neurotrophic factor (*Bdnf*) and Activity-regulated cytoskeleton-associated protein (*Arc*), after a four hour exposure to the commonly used general anaesthetics propofol and sevoflurane. An *in vitro* mouse model was used and each anaesthetic contained a sample size of five mice. Quantitative polymerase chain reaction (qPCR) was used to analyse the changes in gene expression between the four hour exposure samples and time-matched four hour controls.

Our data showed that after a *t=4* hour exposure to propofol, *Bdnf* was down regulated by a factor of 0.710 while *Arc* was up regulated by 1.560. Following a *t=4* hour exposure of sevoflurane, *Bdnf* was up regulated by a factor of 1.859 and Arc was down regulated by 0.348 which was statistically significant ($p<0.05$). Because of the roles that *Bdnf* and *Arc* play in memory consolidation this provides an indication on how synaptic plasticity is affected when the regulation of these two genes changes. With synaptic plasticity being disrupted, this leads to the amnesic effect that is seen with administration of general anaesthetics.

Also the expression differences between an *in vivo* and *in vitro* mouse model was investigated. After a t=4 hour exposure to sevoflurane, *Bdnf* was shown to be down regulated *in vivo* while being upregulated *in vitro*. This may have been due to the differences in tissue preparation of the *in vitro* study and instead of an unknown interaction that is only present *in vivo*. *Arc* was shown to have no change in regulation *in vivo* while *in vitro* had a significant downregulation which agrees with a previous study on sevofluranes interaction with gene expression.

Chapter Six

Future Recommendations

Based on the outcomes of this study, eleven future recommendations have been made and these are discussed in more detail below. They will ultimately depend on budget restrictions.

6.1 Varying Induction and Maintenance Anaesthetics

Both propofol and sevoflurane were used in clinically significant concentrations during this study. During clinical application of general anaesthetics, it is most common to use multiple anaesthetics during an operation. Propofol is often used to induce anaesthesia while sevoflurane is used to maintain anaesthesia. Therefore, to match more closely to a clinical application an *in vivo* gene expression study should be performed that uses propofol and sevoflurane in conjunction.

6.2 Epigenetics

It has been shown that epigenetics plays a role in the expression levels of *Bdnf*. One study found that *Bdnf* was down-regulated after chronic exposure to opiates due to epigenetic modification at specific *Bdnf* gene promoters [111]. Epigenetic differences within a population may affect the expression differences between patients receiving anaesthetics. This could modify their sensitivity to anaesthetics or exacerbate adverse effects such as postoperative cognitive dysfunction. Comparing epigenetic modifications of memory-related genes and their promotors, e.g. *Bdnf*, between mice within a single strain could help elucidate the mechanisms of action for anaesthetic side effects. DNA methylation can be detected using Bisulphite genomic sequencing [112].

6.3 Measure Gene Expression Post-Anaesthetic Exposure

Postoperative cognitive dysfunction (POCD) is a decline in cognitive function that can last from a few days to a few weeks. Symptoms include amnesia, confusion, reduced awareness and attention. This is one of the negative side effects of general anaesthetics. Gene expression analysis should be carried out to analyse the mRNA expression differences 24 hours and 48 hours post anaesthesia. As POCD occurs most commonly in elderly patients, expression differences between young (aged 4 - 8 weeks old) and elderly mice (aged 18 - 22 months) should also be investigated. Alzheimer's disease transgenic mice have been previously used as a model for POCD [113]. An increase in β-amyloid levels in 9-month old Alzheimer's transgenic mice was detected and they also showed cognitive impairment in the Fear Conditioning System (FCS), indicating POCD, while wild-type mice showed no change. Examining the expression differences of memory-related genes between the Alzheimer's transgenic mice and wild-type mice may help elucidate causes of POCD.

6.4 Behavioural Studies

Bdnf expression has been shown to be reduced in patients with depression [114, 115]. Mice involved in gene expression studies that have major depressive disorder could cause a difference in expression results. Behavioural tests can determine the level of depression within a mouse. Forced-swimming test places a mouse inside an inescapable cylinder filled with water. Mice that stop performing escape-directed behaviours and become immobile faster are considered more depressed [116]. Tail suspension test is another depression behavioural test where a mouse is suspended by the tail. Just like the forced-swimming test, the longer the immobility time, the more depressed a mouse is [117]. Using these two tests a level of depression can be recorded and expression analysis can take these into consideration to determine if the behaviour of a mouse interacts with the expression of genes of interest.

6.5 *Bdnf* Knockout Mice

Bdnf has been shown to play a role in activity-dependent synaptic plasticity [49]. *Bdnf* modifies expression of other memory-related genes such as *Arc* and is important in neuronal development and survival [50]. Knockout *Bdnf* die during the perinatal period therefore a heterozygous *Bdnf* mutant would be required [118]. Previous studies have shown that Bdnf expression is required pre- and postnatally for synaptic plasticity and normal behaviour [119]. Heterozygous mutant *Bdnf* mice have severe deficiencies in coordination, balance and show reduced basal synaptic transmission [118, 120]. *Bdnf* mutant mice may have differing pathways to some form of synaptic plasticity and therefore transcriptome analysis should be carried out to compare mutant mice and wild-type mice.

6.6 Investigate Differential mRNA Expression within the Mouse Brain

Expression differences between each sample within an anaesthetic experiment varied greatly. In the propofol experiment there was a range from *Arc* being down regulated by 0.89 to an upregulation of 2.52. This variation could be a result of where the slices were taken from within each brain slice. We attempted to gather posterior sections, but due to our selection criteria this did not always occur and some medial sections were taken. Exploring the gene expression differences between the different areas of the neocortex could uncover whether or not *Bdnf* and *Arc* play a greater role in memory consolidation in certain areas of the brain compared to others.

6.7 Role of Brain Activity

Differences in activity within the mouse brain may effect gene expression levels. Increased SLE activity may indicate more active tissue where the effects of general anaesthetics are amplified. An analysis of gene expression compared to SLE activity should be investigated. If SLE activity does change gene expression then the results of slice studies may be skewed.

6.8 Whole Transcriptome Analysis using RNA-Sequencing

RNA-Seq is a powerful whole transcriptome analysis technique that reveals the quantity of every RNA present within a sample at a given time. RNA-Seq can also elucidate isoforms, splice variants and fusion genes [121]. The use of RNA-Seq in this study would be useful as it could identify other genes, and their isoforms, that have their regulation changed due to the anaesthetic exposure. All of the genes that have variable expression due to anaesthetics could be cross examined to known memory-related genes, such as *Bdnf*, *Arc* or neurotransmitter receptors, to identify genes that have the highest variation. This would help elucidate the reasons for memory loss due to anaesthesia. RNA-Seq is preferred over other transcriptome sequencing platforms, e.g. microarray sequencing, as it provides an unbiased detection of RNAs within the sample due to not requiring transcript specific probes, a wider dynamic range of high-resolution analysis, and the ability to detect rare isoforms. Beijing Genomics Institute (BGI) would be contracted to perform RNA-Seq and bioinformatics analysis [122]. Transcriptomes between single cells have shown to be distinct even within similar tissue [109, 110]. BGI also offers single cell whole transcriptome sequencing, which would allow us to examine expression differences between pre- and post-synaptic neurons and how their expression varies for synaptic plasticity. As this is also whole transcriptome sequencing, novel genes that are related to synaptic plasticity would be elucidated and, therefore, new pathways for anaesthetic application could be explicated.

6.9 Determine Localisation and Level of Protein Expression in the Brain

In this study we attempted to use IHC to analyse the localisation of the Bdnf protein in a coronal brain section and determine if there was a difference in the expression levels. Alternatively, western blots could be used to assess the protein level of Bdnf between an anaesthetics-treated whole mouse brain and an untreated control brain. As mRNA levels do not correlate to protein levels this would allow us to examine if the Bdnf protein levels change in a similar way to the mRNA levels from the results in this study. Mice would be exposed to an anaesthetic for a duration of *t=4 hours*, sacrificed and the whole brain extracted. Whole brain extract could then be run on a 4% - 16% SDS PAGE gel, transferred to a PVDF membrane and subjected to the western blot protocol outlined in section 2.8. A control whole brain extract sample, which has had no anaesthetic exposure, would also be run alongside the treated sample to compare *Bdnf* protein levels to.

6.10 Improve IHC Protocol

IHC is useful to observe where in the brain Bdnf is being expressed with respect to cell type and in what levels compared to other areas of the brain. IHC would also be used to analyse Bdnf protein levels. We were not successful in producing a positive Bdnf protein signal in IHC during this study, however it would be beneficial to improve the IHC protocol to obtain a positive signal and assess the level of Bdnf between an anaesthetics treated brain slice and an untreated control brain slice. To optimise the IHC protocol, antigen retrieval conditions could be changed such as increasing the heating duration, using a steam pressure cooker instead of a microwave, or trialling different pH antigen retrieval buffers like EDTA-NaOH pH 8.0 buffer [123, 124]. A positive antibody control should be used to ensure the protocol is working correctly. Microtubule-associated protein 2 (MAP2) would be an ideal positive control for

the neocortex [125]. Improving fixation times, dehydration and embedding would allow sections to be cut without the tissue tearing or curling. In addition, a normal rabbit serum pre-immune control should be included to demonstrate specificity of the primary antibodies raised in rabbits.

6.11 Arc Antibody

As *Arc* expression has also differed with anaesthetic exposure it would be valuable to source an anti-Arc antibody for use in IHC and western blots. We suggest the Anti-Arg 3.1 antibody (ab23382; Abcam, UK) due to the citations that have managed a positive signal in both western blot and IHC. This Arc antibody would be useful in western blot analysis comparing protein levels between anaesthetic treated and untreated whole mouse brains. It would also be useful in comparing the protein levels within the brain using IHC to the levels shown in Bdnf IHC to elucidate how the Arc and Bdnf protein levels affect each other.

References

1. Greene, N.M., *A consideration of factors in the discovery of anesthesia and their effects on its development.* Anesthesiology, 1971. **35**(5): p. 515-22.

2. Greene, N.M., *Anesthesia and the development of surgery (1846-1896).* Anesthesiology, 1979. **58**(1): p. 5-12.

3. López-Muñoz, F., R. Ucha-Udabe, and C. Alamo, *The history of barbiturates a century after their clinical introduction.* Neuropsychiatric disease and treatment, 2005. **1**(4): p. 329.

4. Robinson, D.H. and A.H. Toledo, *Historical development of modern anesthesia.* J Invest Surg, 2012. **25**(3): p. 141-9.

5. Sahu, D.K., V. Kaul, and R. Parampill, *Comparison of isoflurane and sevoflurane in anaesthesia for day care surgeries using classical laryngeal mask airway.* Indian Journal of Anaesthesia, 2011. **55**(4): p. 364-369.

6. Sloan, M.H., et al., *Sevoflurane versus isoflurane: induction and recovery characteristics with single-breath inhaled inductions of anesthesia.* Anesth Analg, 1996. **82**(3): p. 528-32.

7. Orser, B.A., C.D. Mazer, and A.J. Baker, *Awareness during anesthesia.* CMAJ : Canadian Medical Association Journal, 2008. **178**(2): p. 185-188.

8. Sackel, D.J., *Anesthesia awareness: an analysis of its incidence, the risk factors involved, and prevention.* Journal of Clinical Anesthesia, 2006. **18**(7): p. 483-485.

9. Pan, Z.Q., et al., *The effects of sevoflurane anesthesia on rat hippocampus: A genomic expression analysis.* Brain Research, 2011. **1381**: p. 124-133.

10. Zhiqiang Pan, X.-F.L., Cuijie Shao, Chengbiao Zhang, Junxia Yang, Tao Ma, Li-Cai Zhang, Jun-Li Cao, *The effects of sevoflurane anesthesia on rat hippocampus: A genomic expression analysis.* Brain Research, 2011. **1381**: p. 124-133.

11. Wang, J., et al., *Comparison of the effects of sevoflurane and isoflurane on arterial oxygenation during one lung ventilation.* British journal of anaesthesia, 1998. **81**(6): p. 850-853.

12. Takamatsu, I., et al., *Propofol-mediated impairment of CA1 long-term potentiation in mouse hippocampal slices.* Neuroscience letters, 2005. **389**(3): p. 129-132.

13. Androsova, G., et al., *Biomarkers of postoperative delirium and cognitive dysfunction.* Frontiers in Aging Neuroscience, 2015. **7**: p. 16.

14. Rundshagen, I., *Postoperative Cognitive Dysfunction.* Deutsches Ärzteblatt International, 2014. **111**(8): p. 119-125.

15. Sun, L., *Early childhood general anaesthesia exposure and neurocognitive development.* British Journal of Anaesthesia, 2010. **105**: p. I61-I68.

16. Jevtovic-Todorovic, V., et al., *Anaesthetic neurotoxicity and neuroplasticity: an expert group report and statement based on the BJA Salzburg Seminar.* Br J Anaesth, 2013. **111**(2): p. 143-51.

17. Zhou, Z. and D. Ma, *Anaesthetics-Induced Neurotoxicity in Developing Brain: An Update on Preclinical Evidence.* Brain Sciences, 2014. **4**(1): p. 136-149.

18. Snell, R.S., *Clinical Neuroanatomy*. 7 ed. 2009: Lippincott Williams and Wilkins. 285-286.

19. Bear, M.F., *A synaptic basis for memory storage in the cerebral cortex.* Proceedings of the National Academy of Sciences, 1996. **93**: p. 13453-13459.

20. Herrup, K. and Y. Yang, *Cell cycle regulation in the postmitotic neuron: oxymoron or new biology?* Nat Rev Neurosci, 2007. **8**(5): p. 368-378.

21. Williams, R.W. and K. Herrup, *The control of neuron number.* Annu Rev Neurosci, 1988. **11**: p. 423-53.

22. Bramham, C.R., et al., *The Arc of synaptic memory.* Exp Brain Res, 2010. **200**(2): p. 125-40.

23. Perez-Cadahia, B., B. Drobic, and J.R. Davie, *Activation and function of immediate-early genes in the nervous system.* Biochem Cell Biol, 2011. **89**(1): p. 61-73.

24. Gerrow, K. and A. Triller, *Synaptic stability and plasticity in a floating world.* Curr Opin Neurobiol, 2010. **20**(5): p. 631-9.

25. Miyashita, T., et al., *Networks of Neurons, Networks of Genes: An Integrated View of Memory Consolidation.* Neurobiology of learning and memory, 2008. **89**(3): p. 269-284.

26. Malenka, R.C. and M.F. Bear, *LTP and LTD: an embarrassment of riches.* Neuron, 2004. **44**(1): p. 5-21.

27. Shipp, S., *Structure and function of the cerebral cortex.* Current Biology, 2007. **17**(12): p. 443-449.

28. Bentivoglio, M. *Life and Discoveries of Santiago Ramón y Cajal*. [cited 2016 9th Feburary]; Available from: http://www.nobelprize.org/nobel_prizes/medicine/laureates/1906/cajal-article.html.

29. Ranganath, C. and R.S. Blumenfeld, *Doubts about double dissociations between short-and long-term memory.* Trends in cognitive sciences, 2005. **9**(8): p. 374-380.

30. Cajal, S.R., *Comparative study of the sensory areas of the human cortex*. 1899.

31. Fulton, B. and E.M. Sorkin, *Propofol.* Drugs, 1995. **50**(4): p. 636-457.

32. Trapani, G., et al., *Propofol in anesthesia. Mechanism of action, structure-activity relationships, and drug delivery.* Current medicinal chemistry, 2000. **7**(2): p. 249-271.

33. Krasowski, M.D., et al., *4D-QSAR analysis of a set of propofol analogues: mapping binding sites for an anesthetic phenol on the GABAA receptor.* Journal of medicinal chemistry, 2002. **45**(15): p. 3210-3221.

34. Gredell, J.A., et al., *Determination of diffusion and partition coefficients of propofol in rat brain tissue: implications for studies of drug action in vitro.* Br J Anaesth, 2004. **93**(6): p. 810-7.

35. II, E.I.E., *New Inhaled Anesthetics.* Anesthesiology, 1994. **80**: p. 906-922.

36. http://livertox.nlm.nih.gov/Sevoflurane.htm. *OVERVIEW: Sevoflurane*. [cited October 2014.

37. Hapfelmeier, G., H. Schneck, and E. Kochs, *Sevoflurane potentiates and blocks GABA‐induced currents through recombinant $\alpha 1\beta 2\gamma 2$ GABAA receptors: implications for an enhanced GABAergic transmission.* European journal of anaesthesiology, 2001. **18**(6): p. 377-383.

38. Swearengin, T.A., E.E. Fibuch, and N.W. Seidler, *Sevoflurane modulates the activity of glyceraldehyde 3-phosphate dehydrogenase.* Journal of enzyme inhibition and medicinal chemistry, 2006. **21**(5): p. 575-579.

39. Sheng Peng, Y.Z., Da-Peng Sun, Deng-Xin Zhang, Qiang Fang, Guo-Jun Li, *The effect of sevoflurane anesthesia on cognitive function and the expression of Insulin-like Growth Factor-1 in CA1 region of hippocampus in old rats.* Molecular Biology Reports, 2011. **38**: p. 1195-1199.

40. Aid, T., et al., *Mouse and rat BDNF gene structure and expression revisited.* Journal of neuroscience research, 2007. **85**(3): p. 525-535.

41. Sakata, K., et al., *Role of activity-dependent BDNF expression in hippocampal–prefrontal cortical regulation of behavioral perseverance.* Proceedings of the National Academy of Sciences, 2013. **110**(37): p. 15103-15108.

42. Huang, E.J. and L.F. Reichardt, *Neurotrophins: roles in neuronal development and function.* Annu Rev Neurosci, 2001. **24**: p. 677-736.

43. Ernfors, P., et al., *Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice.* Int J Dev Biol, 1995. **39**(5): p. 799-807.

44. Acheson, A., et al., *A BDNF autocrine loop in adult sensory neurons prevents cell death.* Nature, 1995. **374**(6521): p. 450-3.

45. Yamada, K. and T. Nabeshima, *Brain-derived neurotrophic factor/TrkB signaling in memory processes.* J Pharmacol Sci, 2003. **91**(4): p. 267-70.

46. Patapoutian, A. and L.F. Reichardt, *Trk receptors: mediators of neurotrophin action.* Curr Opin Neurobiol, 2001. **11**(3): p. 272-80.

47. Lu, L.X., et al., *General anesthesia activates BDNF-dependent neuroapoptosis in the developing rat brain.* Apoptosis, 2006. **11**(9): p. 1603-15.

48. Santos, A.R., D. Comprido, and C.B. Duarte, *Regulation of local translation at the synapse by BDNF.* Progress in neurobiology, 2010. **92**(4): p. 505-516.

49. Yin, Y., G.M. Edelman, and P.W. Vanderklish, *The brain-derived neurotrophic factor enhances synthesis of Arc in synaptoneurosomes.* Proceedings of the National Academy of Sciences, 2002. **99**(4): p. 2368-2373.

50. Alder, J., et al., *Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity.* The Journal of neuroscience, 2003. **23**(34): p. 10800-10808.

51. Link, W., et al., *Somatodendritic expression of an immediate early gene is regulated by synaptic activity.* Proceedings of the National Academy of Sciences, 1995. **92**(12): p. 5734-5738.

52. Lyford, G.L., et al., *Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites.* Neuron, 1995. **14**(2): p. 433-445.

53. Kubik, S., T. Miyashita, and J.F. Guzowski, *Using immediate-early genes to map hippocampal subregional functions.* Learning & Memory, 2007. **14**(11): p. 758-770.

54. Kawashima, T., et al., *Synaptic activity-responsive element in the Arc/Arg3. 1 promoter essential for synapse-to-nucleus signaling in activated neurons.* Proceedings of the National Academy of Sciences, 2009. **106**(1): p. 316-321.

55. Waltereit, R., et al., *Arg3. 1/Arc mRNA induction by Ca2+ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation.* The Journal of Neuroscience, 2001. **21**(15): p. 5484-5493.

56. Pintchovski, S.A., et al., *The serum response factor and a putative novel transcription factor regulate expression of the immediate-early gene Arc/Arg3. 1 in neurons.* The Journal of Neuroscience, 2009. **29**(5): p. 1525-1537.

57. Kobayashi, H., et al., *Identification of a cis‐acting element required for dendritic targeting of activity‐regulated cytoskeleton‐associated protein mRNA.* European Journal of Neuroscience, 2005. **22**(12): p. 2977-2984.

58. Gao, Y., et al., *Multiplexed dendritic targeting of α calcium calmodulin-dependent protein kinase II, neurogranin, and activity-regulated cytoskeleton-associated protein RNAs by the A2 pathway.* Molecular biology of the cell, 2008. **19**(5): p. 2311-2327.

59. Dynes, J.L. and O. Steward, *Dynamics of bidirectional transport of Arc mRNA in neuronal dendrites.* Journal of Comparative Neurology, 2007. **500**(3): p. 433-447.

60. Rao, V.R., et al., *AMPA receptors regulate transcription of the plasticity-related immediate-early gene Arc.* Nature neuroscience, 2006. **9**(7): p. 887-895.

61. Messaoudi, E., et al., *Sustained Arc/Arg3. 1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo.* The Journal of Neuroscience, 2007. **27**(39): p. 10445-10455.

62. Waung, M.W., et al., *Rapid translation of Arc/Arg3. 1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate.* Neuron, 2008. **59**(1): p. 84-97.

63. Plath, N., et al., *Arc/Arg3. 1 is essential for the consolidation of synaptic plasticity and memories.* Neuron, 2006. **52**(3): p. 437-444.

64. Guzowski, J.F., et al., *Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory.* The Journal of neuroscience, 2000. **20**(11): p. 3993-4001.

65. Kobayashi, K., K. Takemori, and A. Sakamoto, *Circadian gene expression is suppressed during sevoflurane anesthesia and the suppression persists after awakening.* Brain research, 2007. **1185**: p. 1-7.

66. Lectures, N. *The Polymerase Chain Reaction*. 1993 [cited 2016 9th Feburary]; Available from: http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1993/mullis-lecture.html.

67. Bartlett, J.M. and D. Stirling, *A short history of the polymerase chain reaction*, in *PCR protocols*. 2003, Springer. p. 3-6.

68. Stephen A. Bustin, V.B., Jeremy A. Garson, Jan Hellemans, Jim Huggett, Mikael Kubista, Reinhold Mueller, Tania Nolan, Michael W. Pfaffl, Gregory L. Shipley, Jo Vandesompele, Carl T. Wittwer, *The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.* Clinical Chemistry, 2009. **55**(4): p. 611-622.

69. van Rijn, S.J., et al., *Expression stability of reference genes for quantitative RT-PCR of healthy and diseased pituitary tissue samples varies between humans, mice, and dogs.* Molecular neurobiology, 2014. **49**(2): p. 893-899.

70. Chinwalla, A.T., et al., *Initial sequencing and comparative analysis of the mouse genome.* Nature, 2002. **420**(6915): p. 520-562.

71. Laboratory, T.J. *The Jackson Laboratory*. 2016 [cited 2016 9th Feburary]; Available from: https://www.jax.org/#.

72. Voss, L.J., et al., *Role of Cx36 gap junction modulation in general anaesthetic anticonvulsant action.* European journal of pharmacology, 2010. **643**(1): p. 58-62.

73. Voss, L.J. and J.W. Sleigh, *Cortico-centric effects of general anesthetics on cerebrocortical evoked potentials.* Neuroscience bulletin, 2015. **31**(6): p. 697-704.

74. NCBI. *GenBank*. 2016 [cited 2016 9th Feburary]; Available from: http://www.ncbi.nlm.nih.gov/genbank/.

75. GenBank. *Mus musculus beta-2 microglobulin (B2m), mRNA*. 2016 [cited 2016 14th Feburary]; Available from: http://www.ncbi.nlm.nih.gov/nuccore/144227219/#.

76. GenBank. *Mus musculus glyceraldehyde-3-phosphate dehydrogenase (Gapdh), transcript variant 1, mRNA*. 2016 [cited 2016 14th Feburary]; Available from: http://www.ncbi.nlm.nih.gov/nuccore/576080554/.

77. Xia, L., et al., *Ventral hippocampal molecular pathways and impaired neurogenesis associated with 5-HT 1A and 5-HT 1B receptors disruption in mice.* Neuroscience letters, 2012. **521**(1): p. 20-25.

78. GenBank. *Mus musculus growth factor ARC (Arc) mRNA, complete cds*. 2016 [cited 2016 14th Feburary]; Available from: http://www.ncbi.nlm.nih.gov/nuccore/5453466/.

79. Gilsbach, R., et al., *Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data.* Biotechniques, 2006. **40**(2): p. 173.

80. Wang, Y., et al., *Neuronal gap junction coupling is regulated by glutamate and plays critical role in cell death during neuronal injury.* The Journal of Neuroscience, 2012. **32**(2): p. 713-725.

81. Charan, J. and N.D. Kantharia, *How to calculate sample size in animal studies?* Journal of Pharmacology & Pharmacotherapeutics, 2013. **4**(4): p. 303-306.

82. Schroeder, A., et al., *The RIN: an RNA integrity number for assigning integrity values to RNA measurements.* BMC molecular biology, 2006. **7**(1): p. 3.

83. Aranda, P.S., D.M. LaJoie, and C.L. Jorcyk, *Bleach gel: a simple agarose gel for analyzing RNA quality.* Electrophoresis, 2012. **33**(2): p. 366-369.

84. Björkman, J., et al., *Differential amplicons (ΔAmp)—a new molecular method to assess RNA integrity.* Biomolecular Detection and Quantification, 2016. **6**: p. 4-12.

85. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method.* methods, 2001. **25**(4): p. 402-408.

86. Radonić, A., et al., *Guideline to reference gene selection for quantitative real-time PCR.* Biochemical and biophysical research communications, 2004. **313**(4): p. 856-862.

87. Ray, M.T., C. Shannon Weickert, and M.J. Webster, *Decreased BDNF and TrkB mRNA expression in multiple cortical areas of patients with schizophrenia and mood disorders.* Translational Psychiatry, 2014. **4**(5): p. e389.

88. Xiong, P., et al., *Combining serum protein concentrations to diagnose schizophrenia: a preliminary exploration.* The Journal of clinical psychiatry, 2014. **75**(8): p. 1,478-801.

89. Warner‐Schmidt, J.L. and R.S. Duman, *Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment.* Hippocampus, 2006. **16**(3): p. 239-249.

90. Mattson, M.P., *Glutamate and Neurotrophic Factors in Neuronal Plasticity and Disease.* Annals of the New York Academy of Sciences, 2008. **1144**: p. 97-112.

91. Berninger, B., et al., *GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation in vitro.* Development, 1995. **121**(8): p. 2327-35.

92. Vaidya, V.A., R.M. Terwilliger, and R.S. Duman, *Role of 5-HT2A receptors in the stress-induced down-regulation of brain-derived neurotrophic factor expression in rat hippocampus.* Neurosci Lett, 1999. **262**(1): p. 1-4.

93. Vaidya, V.A., et al., *5-HT2A receptor-mediated regulation of brain-derived neurotrophic factor mRNA in the hippocampus and the neocortex.* J Neurosci, 1997. **17**(8): p. 2785-95.

94. Zetterstrom, T.S., et al., *Manipulations of brain 5-HT levels affect gene expression for BDNF in rat brain.* Neuropharmacology, 1999. **38**(7): p. 1063-73.

95. Slack, S.E., et al., *Brain-derived neurotrophic factor induces NMDA receptor subunit one phosphorylation via ERK and PKC in the rat spinal cord.* Eur J Neurosci, 2004. **20**(7): p. 1769-78.

96. Tezuka, T., et al., *PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 435-40.

97. Mizuno, M., et al., *Involvement of BDNF receptor TrkB in spatial memory formation.* Learn Mem, 2003. **10**(2): p. 108-15.

98. Caldeira, M.V., et al., *Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons.* J Biol Chem, 2007. **282**(17): p. 12619-28.

99. Wu, K., et al., *Brain-derived neurotrophic factor acutely enhances tyrosine phosphorylation of the AMPA receptor subunit GluR1 via NMDA receptor-dependent mechanisms.* Brain Res Mol Brain Res, 2004. **130**(1-2): p. 178-86.

100. Gall, C., et al., *Seizures and the regulation of neurotrophic factor and neuropeptide gene expression in brain.* Epilepsy Res Suppl, 1991. **4**: p. 225-45.

101. Gautam, A., R. Wadhwa, and M.K. Thakur, *Involvement of hippocampal Arc in amnesia and its recovery by alcoholic extract of Ashwagandha leaves.* Neurobiology of learning and memory, 2013. **106**: p. 177-184.

102. Wang, D.-C., et al., *Amyloid-β at sublethal level impairs BDNF-induced arc expression in cortical neurons.* Neuroscience letters, 2006. **398**(1): p. 78-82.

103. Giorgi, C., et al., *The EJC factor eIF4AIII modulates synaptic strength and neuronal protein expression.* Cell, 2007. **130**(1): p. 179-191.

104. Steward, O. and P.F. Worley, *Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation.* Neuron, 2001. **30**(1): p. 227-240.

105. Granado, N., et al., *D1 but not D5 dopamine receptors are critical for LTP, spatial learning, and LTP-Induced arc and zif268 expression in the hippocampus.* Cerebral cortex, 2008. **18**(1): p. 1-12.

106. Bloomer, W.A., H.M. VanDongen, and A.M. VanDongen, *Arc/Arg3. 1 translation is controlled by convergent N-methyl-D-aspartate and Gs-coupled receptor signaling pathways.* Journal of Biological Chemistry, 2008. **283**(1): p. 582-592.

107. Doherty, G.J. and H.T. McMahon, *Mediation, modulation, and consequences of membrane-cytoskeleton interactions.* Annu Rev Biophys, 2008. **37**: p. 65-95.

108. Harris, K.M., *Structure, development, and plasticity of dendritic spines.* Current opinion in neurobiology, 1999. **9**(3): p. 343-348.

109. Darmanis, S., et al., *A survey of human brain transcriptome diversity at the single cell level.* Proceedings of the National Academy of Sciences, 2015. **112**(23): p. 7285-7290.

110. Tang, F., K. Lao, and M.A. Surani, *Development and applications of single cell transcriptome analysis.* Nature methods, 2011. **8**(4 Suppl): p. S6-11.

111. Koo, J.W., et al., *Epigenetic basis of opiate suppression of Bdnf gene expression in the ventral tegmental area.* Nat Neurosci, 2015. **18**(3): p. 415-22.

112. Li, Y. and T.O. Tollefsbol, *DNA methylation detection: bisulfite genomic sequencing analysis.* Epigenetics Protocols, 2011: p. 11-21.

113. Xu, Z., et al., *Age-dependent postoperative cognitive impairment and Alzheimer-related neuropathology in mice.* Sci Rep, 2014. **4**: p. 3766.

114. Lee, B.-H. and Y.-K. Kim, *The Roles of BDNF in the Pathophysiology of Major Depression and in Antidepressant Treatment.* Psychiatry Investigation, 2010. **7**(4): p. 231-235.

115. Castren, E. and T. Rantamaki, *The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity.* Dev Neurobiol, 2010. **70**(5): p. 289-97.

116. Petit-Demouliere, B., F. Chenu, and M. Bourin, *Forced swimming test in mice: a review of antidepressant activity.* Psychopharmacology (Berl), 2005. **177**(3): p. 245-55.

117. Cryan, J.F., C. Mombereau, and A. Vassout, *The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice.* Neurosci Biobehav Rev, 2005. **29**(4-5): p. 571-625.

118. Ernfors, P., K.F. Lee, and R. Jaenisch, *Mice lacking brain-derived neurotrophic factor develop with sensory deficits.* Nature, 1994. **368**(6467): p. 147-50.

119. Chan, J.P., et al., *Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice.* Neuroscience, 2006. **142**(1): p. 49-58.

120. Patterson, S.L., et al., *Recombinant BDNF Rescues Deficits in Basal Synaptic Transmission and Hippocampal LTP in BDNF Knockout Mice.* Neuron, 1996. **16**(6): p. 1137-1145.

121. Wang, Z., M. Gerstein, and M. Snyder, *RNA-Seq: a revolutionary tool for transcriptomics.* Nature reviews. Genetics, 2009. **10**(1): p. 57-63.

122. Institute, B.G. *RNA-Seq*. 2016 [cited 2016 2nd Feburary]; Available from: http://bgi-international.com/services/genomics/rna-seqtranscriptome/.

123. Shi, S.-R., R.J. Cote, and C.R. Taylor, *Antigen Retrieval Techniques: Current Perspectives.* Journal of Histochemistry & Cytochemistry, 2001. **49**(8): p. 931-937.

124. Shi, S.-R., Y. Shi, and C.R. Taylor, *Antigen Retrieval Immunohistochemistry: Review and Future Prospects in Research and Diagnosis over Two Decades.* Journal of Histochemistry and Cytochemistry, 2011. **59**(1): p. 13-32.

125. Lyck, L., et al., *Immunohistochemical Markers for Quantitative Studies of Neurons and Glia in Human Neocortex.* Journal of Histochemistry and Cytochemistry, 2008. **56**(3): p. 201-221.

Appendices

Appendix 1: Reagents and Solutions

Agarose (1%) Gel with 1X TAE buffer

To prepare a 1% agarose gel with 1X TAE buffer, add 0.4 g of agarose to 40 mL of 1X TAE and dissolve by heating in a microwave oven. When the solution has cooled to 60°C, add 1 µL of 10 mg/mL EtBr and gently mix before pouring into the level gel tank. Leave for at least 30 minutes to set.

DEPC H_2O

To prepare 0.1% DEPC H_2O, add 2 mL of DEPC to 2000 mL of sterile mQH_2O in the fume hood. The solution should be mixed with a magnetic stirrer in the fume hood overnight to ensure all DEPC is completely dissolved. It is important to wear gloves and safety glasses and use the fume hood when making this solution, as DEPC is a suspected carcinogen. Once the DEPC has been dissolved overnight, autoclave the solution on a fluids cycle. Store at RT.

4% Paraformaldehyde

To 16 mL of mQH_2O add 2g Paraformaldehyde stock powder and 6 µL of 10 M NaOH. Dissolve for one hour in a 60 °C water bath. After completely dissolved, add 10 mL of 10X PBS and 10 µL 10 M HCl then adjust to pH 7.4. Fill to 50 mL with mQH_2O. Filter solution through a 0.45 µm membrane filter. Store at 4 °C for up to one month.

EDTA (0.5 M)

To prepare EDTA at 0.5 M, add 186.1 g of disodium EDTA to 800 mL of mQH_2O. Stir vigorously using a magnetic stirrer. Adjust pH to desired value with NaOH. Dispense into aliquots and sterilise by autoclaving.

TAE Buffer (1X)

To prepare a 50X stock solution of TAE buffer, add 242 g of Tris and 18.61 g of disodium EDTA to approximately 700 mL mQH_2O and stir until dissolved. Add the acetic acid and adjust volume to 1 L. To make a 1X working buffer, add 20 mL of the stock solution to 980 mL of mQH_2O.

Tris-Cl (1M)

To prepare a 1M Tris-Cl solution, dissolve 121.1 g of Tris base in 800 mL of mQH_2O. Adjust pH to desired value with HCl.

Tris EDTA (TE) Buffer

To prepare Tris EDTA buffer, add 2 mL of Tris (1 M) and 4 mL of EDTA (0.5 M) to a sterile bottle. Make up to 200 mL with sterile mQH_2O and mix by gentle inversion. Store at RT.

PBS Buffer (1X)

To prepare a 10X stock solution of PBS buffer, add 80 g of NaCl, 2 g of KCl, 14.2 g Na_2HPO_4 and 2.4 g of KH_2PO_4 to 800 mL of mQH_2O. Adjust pH to 7.4 with HCl and then fill to 1 L with mQH_2O. To make a 1X working buffer, add 100 mL of 10X PBS to 900 mL of mQH_2O

PBS-T Buffer (1X)

To make a 1X working buffer, add 100 mL of 10X PBS and 1 mL Tween 20 to 899 mL of mQH_2O. Stir on magnetic stirrer until Tween 20 completely dissolved.

TBS Buffer (1X)

To prepare a 10X stock solution of TBS buffer, add 60.5 g Tris, 87.6 g NaCl to 800 mL of mQH_2O. Adjust pH to 7.4 with HCl and then fill to 1 L with mQH_2O. To make a 1X working buffer, add 100 mL of 10X TBS to 900 mL of mQH_2O

TBS-T Buffer (1X)

To make a 1X working buffer, add 100 mL of 10X TBS and 1 mL Tween 20 to 899 mL of mQH_2O. Stir on magnetic stirrer until Tween 20 completely dissolved.

Laemmli Sample Buffer (2X)

To prepare a 2X working Laemmli sample buffer add 1 mL 1M Tris-HCl, 0.4 g SDS, and 2 mL 100% glycerol. Fill up to 9 mL with mQH_2O. Add 10% β-mercaptoethanol freshly before use.

DNA Loading Buffer (6X)

To a 15 mL Falcon tube, add 3 mL of 100% glycerol, 25 mg of bromophenol blue and 25 mL of xylene cyanol. Fill up to 10 mL with mQH_2O.

Appendix 2: Animal Ethics Approval

UNIVERSITY OF WAIKATO ANIMAL ETHICS COMMITTEE

THE UNIVERSITY OF WAIKATO

Protocol Number: 905

APPLICATIONCOVER SHEET

Project Details
Full Protocol Title: General anaesthetic modulation of memory-related gene expression in the mouse brain **Name of Primary Applicant**: Laura Bell **Faculty/School/Department:** Department of Biological Sciences **Expected start date**: Nov 2013 **Expected completion date**: Nov 2015 **Animals species:** Mouse **Number to be used:** 50 (common name) **Impact Level:**B (See Q 6 Animal Use Statistics Form – Appendix 1):

Type of Application (Can tick more than one box):	√Research √Part of research thesis (Laura Bell) ☐Teaching ☐Other (Specify)
Standard Operating Procedures:	☐ No √ Yes: SOPNumber/ Title: #9 Euthanasia of rodents by CO_2 asphyxiation #5 The Housing and Care of Laboratory Rodents
Other AEC approval:	Has this application been submitted any other AEC for approval √ No ☐ Yes (Specify Committee) Details:
Funding support:	Is this research part of a funding grant either received or pending √ No ☐ Yes (Specify funding source) Details:

OFFICE USE ONLY This proposal is approved for the period:	***Protocol Number:***
From: 1 Nov 2013	**To:** 1 Nov 2015
Signature AEC Chair:	**Date:** 25/10/13

All research involving the use of animals must comply with the *Animal Welfare Act (1999)* and the University of Waikato Code of Ethical Conduct for the Use of Animals in Teaching and Research.

Appendix 3: Waikato Safety Operating Procedure (SOP) 9

UNIVERSITY OF WAIKATO ANIMAL ETHICS COMMITTEE

STANDARD OPERATING PROCEDURE

SOP Number: 9

Title: Euthanasia of Rodents by CO2 Asphyxiation

Only persons that have received appropriate training and have been approved by the Animal Ethics Committee may perform this euthanasia

General:

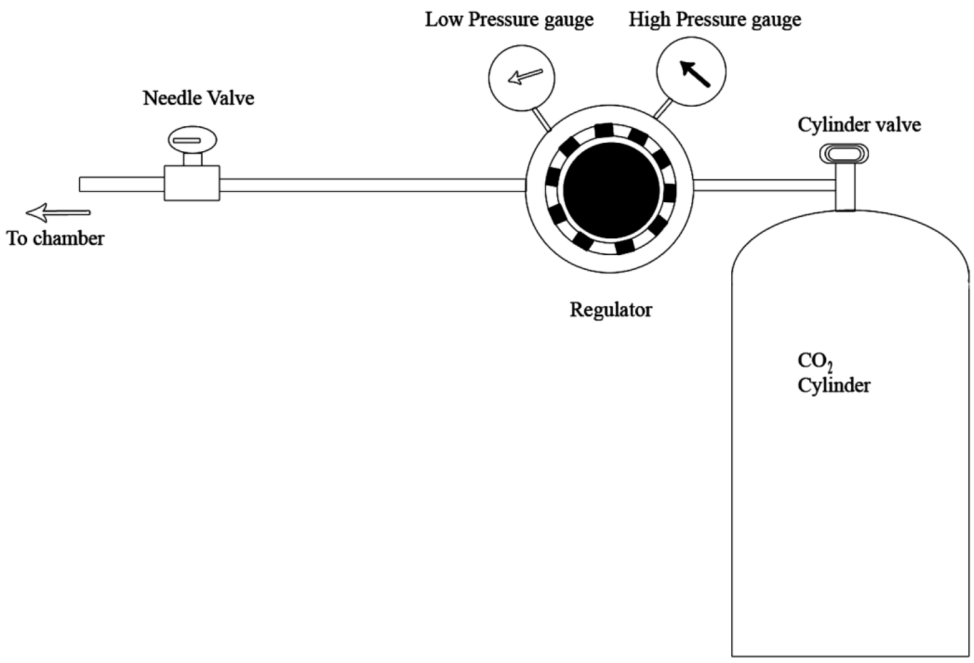
This procedure outlines the general procedure for euthanizing rodents using CO2 gas.

Equipment Required:

Building Animal House (Glasshouse compound) has a purpose built chamber connected to a CO2 cylinder.

Setup:

- Check with the Technician if there are any questions or concerns.
- Put the cage still containing the animal(s) directly in chamber. It is recommended that animals be euthanised in their “home” cages. There has been less stress observed if the animal(s) are with familiar surroundings and smells.
- Ensure that the chamber lid is shut
- It is essential that there is adequate ventilation for the operator.
- Operation (see diagram):
    1. Ensure needle valve is closed
    2. Open cylinder valve one full turn
    3. Turn regulator until low pressure gauge reads “200”
    4. Slowly open needle valve 2 turns



- Once the concentration has caused the animal(s) to stop breathing, the CO2 may be turned off but the chamber should remain closed for 10 minutes.
    1. Turn off cylinder valve
    2. Wait for pressure to drop
    3. Loosen off the regulator
    4. Close needle valve
- After 10 minutes open the chamber and ensure that the animals are dead. There should be an absence of breathing, no detectable heartbeat and glazed over eyes.

The chamber has a quite large volume and it may take some time for the level of CO2 to rise up to and into the cage tray.

Alternatives:

Small numbers of animals:

Where there are small numbers of animals involved, an effective method is to place the cage into a plastic bag and insert the gas hose into the bag and

seal it off. Single animals maybe euthanised in a clear lidded bucket that has the gas hose inserted. Food wrap or a clear plastic bag over the top works well.

Dealing with large numbers of animals:

The chamber can only fit a limited number of cages at once.

The operator:

1. can rotate the cages through (but chamber must be vented between batches; it is painful for the animals to be dunked straight into high CO2 concentrations (ANZCART, 2006).
2. transfer the animals to as few cages as possible; operator must carefully check that the animals are all dead. Ones at the bottom of the pile may lie in air pockets
3. let all animals run loose in the chamber (without cages). They will be too busy sniffing each other to notice the effects of the gas. The operator will then have to clean up the chamber afterward.

Field work with wild animals:

The chamber in the Animal House is not portable; therefore field euthanasia will have to involve the use of a small CO2 cylinder, regulator, needle valve and plastic bag or a custom made chamber (or tube). Use a dark bag so not to stress out the animal further. Wild animal respond differently to CO2 and may take longer to succumb than lab bred animals. DO NOT bring wild animals into Animal House for euthanizing without permission from the technician; wild animals carry diseases/parasites which could transmit to lab animals being housed in Animal House.

Adverse Events and Unexpected Outcomes: Occasionally animals have recovered consciousness and/or exhibited signs of life sometime after the procedure. They were not fully euthanised but the operator did not observe signs of life (wait the full 10 minutes). This possibly is due to the animal(s) being at the bottom of a pile and in a lower (insufficient) concentration of CO2. The animal(s) must be returned to the chamber (or bucket) without delay to complete the euthanasia.

Occupational Safety and Health Considerations: CO2 gas is heavier than air. In confined spaces the gas displaces air and could cause asphyxiation to the operator in high concentrations. Ensuring ventilation during these operations is essential (i.e. open the doors and remain away from the chamber when possible, preferably outside). Asphyxiation symptoms may not be apparent and their onset may be quick. Refer to the MSDS for more information.

Reference:

ANZCART, 2006. Report of the International Consensus Meeting on Carbon Dioxide Euthanasia of Laboratory Animals. ANZCART News 19:2 1-7 summarized from the final report prepared by Hawkins, P et al. Copy available from: (www.nc3rs.org.uk/CO2ConsensusReport).

Appendix 4: Raw Expression Data

All raw qPCR data, expression calculations and statistical analysis are on the accompanying disc.