Prifysgol Abertawe
Swansea University

Faculty of Medicine, Health and Life Science

Lumbriculus variegatus: A novel organism for *in vivo* pharmacology research

By

Caitlin Bellamy, BSc Hons

MSc in Medical and Health Care Studies by Research

SWIRL
Swansea Worm Integrative Research Laboratory

Declaration and statements

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Abstract

For years animal models in science have been invaluable and highly beneficial to the advancement of medicine and pharmacology. Many *in vivo* models are protected by the Animals (Scientific Procedures) Act 1986, and there is framework put in place to replace, reduce and refine the number of animals used in research. This means there is a call for an *in vivo* model that will give us insight into specific pharmacological processes, while reducing the need for vertebrate animal models in research. Here we present the fresh freshwater invertebrate, *Lumbriculus variegatus*, as a novel *in vivo* model for pharmacology research.

Here we have developed two assays to measure the behavioural effects of drugs on *L. variegatus* when exposed to specific compounds: the stereotypical movement assay, which measures the worms stereotyped behaviours in response to stimuli, and the free locomotion assay, which measures *L. variegatus* unstimulated movement. We report the effects of compounds with diverse pharmacodynamic properties on *L. variegatus* using these assays, these include ion channel blockers, neurotransmitters and their antagonists, and drugs of abuse. Alongside this, we have also developed techniques to extract and quantify protein and DNA from this organism.

Our results show that ion channel blockers, lidocaine and quinine, reduced both stimulated and unstimulated movement in *L. variegatus*. Stereotypical movement and free locomotion were both significantly affected when *L. variegatus* were exposed to ≥20 mM of dopamine and ≥50 µM of dopamine antagonist haloperidol. However, dose-dependent effects were only observed for stimulated movement when exposed to GABA, and changes were observed only at the highest concentration of 500 mM when exposed to glycine. Both stimulated and unstimulated movement was reduced when *L. variegatus* was exposed to ≥250 mM. *L. variegatus* also displayed a dose-dependent response to DNP and were unable to recover after 24 hours at 50 µM. These toxic effects were reversed by 10 and 25 µM of haloperidol, and 25 µM of sulpiride. We successfully extracted and quantified both protein and DNA from this organism.

We recognise that the experiments we have conducted on *L. variegatus* throughout this project may not replicate the complexity of higher animals, and experiments utilising invertebrates will not fully replace studies in vertebrate species. *L. variegatus* have the potential to replace smaller invertebrate models where specialist equipment is needed to visualise them. An advantage of using *L. variegatus* for pharmacology is that they possess unique stereotypical behaviours that can be easily quantified without the need for specialist equipment. Alongside this, there is no call for special husbandry as with rodents and other larger models, therefore *L. variegatus* can be cultured in most laboratories, including research and educational institutions.

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Publications and Presentations

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  https://doi.org/10.1002/prp2.853
- From Explosives to Diet Pills: DNP Poisoning in Wales
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  From explosives to diet pills: DNP poisoning in Wales | British Pharmacological Society (bps.ac.uk)
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  Presented at BPS Pharmacology 2022

Abbreviations

ABPI	Association of the British Pharmaceutical Industry
ADME	Administration, distribution, metabolism and excretion
ADH	Alcohol dehydrogenase
AFT	Acute functional tolerance
ASPA	Animals (Scientific Procedures) Act 1986
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
COSHH	Control of substances hazardous to health
DNA	Deoxyribonucleic acid
DBV	Dorsal blood vessel
DMSO	Dimethyl sulfoxide
DNP	2,4-dinitrophenol
ERASMUS	Effective, Rational, Adjusted, Safe and Monitored Use
GABA	Gama-aminobutyric acid
GEM	Genetically engineered models
GLP	Good laboratory practice
GlyRs	Glycine receptors
GPCRs	G-protein coupled receptors
HPV	Human papilloma virus
IAPs	Inhibitors of apoptosis proteins
LD_{50}	Lethal dose
LGF	Lateral giant fibre
LOAEL	Lowest observable adverse effect level
MCR	Mitochondrial respiratory chain
MGF	Medial giant fibre
nACH	Nicotinic acetylcholine receptors
NMDARs	Glutamate-gated channels
NOAEL	No observable adverse effect level
NPIS	The National Poisons Information Service
OXPHOS	Oxidative phosphorylation

PCR	Polymerase Chain Reaction
PIC	Protease inhibitor cocktail
PNS	Peripheral nervous system
RIPA	Radio immunoprecipitation assay
RNAi	Ribonucleic acid interference
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
UCP1	Uncoupling protein 1
VBV	Ventral blood vessel

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1. Introduction

It is a fundamental goal in biomedicine that we understand, treat and prevent diseases (Hartung, 2013). To treat and prevent such diseases, we must be able to develop safe and effective innovative medicines, control the safety of medicines for both humans and animals, and facilitate innovation and research (Orme et al., 2010). While there are adequate therapeutic options available for most human diseases, some have limited or less effective treatment options, these include diabetes, Alzheimer's disease, human immunodeficiency virus-associated acquired immune deficiency syndrome (HIV-AIDS), neglected tropical diseases and rare diseases (Kiriiri et al., 2020). However, before we can treat and prevent, we must understand disease pathology, progression and treatment. Animal models have been used to mimic human diseases and disorders, to limit the risk to human life (Simmons, 2008). Although animals are permitted for drug development, in bioassay and for both general and toxicity preclinical testing (Badyal & Desai, 2014), there is framework in place to protect and reduce the number of animals used in research (Tannenbaum & Bennett, 2015), such as Russel & Burch's 3Rs and the Animals (Scientific Procedures) Act 1986 (ASPA).

Drug discovery and development are complicated and difficult processes that commences when disease, or disease with unmet clinical need is identified (Kiriiri et al., 2020). It can take on average 10-15 years, consists of several stages (Brake et al., 2017), as outlined in Figure 1.1., and can cost an estimated £1.15 billion per new drug developed (Torjesen, 2015).

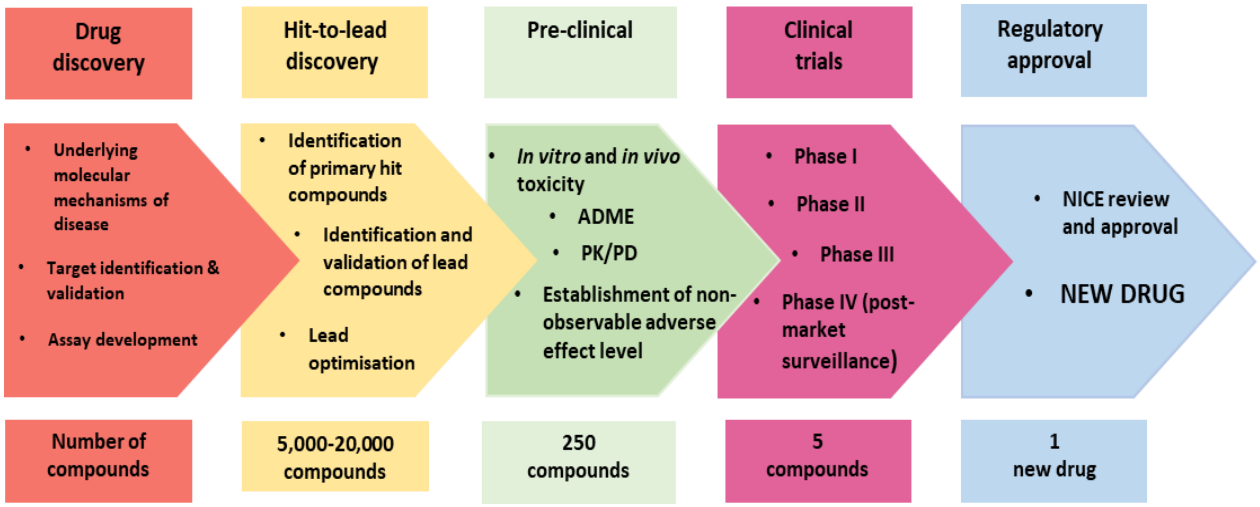

Figure 1.1. Drug discovery pipeline. Flow chart illustrating each stage of drug discovery and development, what processes are included in each, and how many compounds are involved in each stage. Diagram adapted from Matthews et al. (2016) and Kiriiri et al. (2020). Drug discovery, or target identification and validation, is pinpointing and understanding the role of a potential biological target in disease pathology (Lansdowne, 2022). At the point of target identification and validation, hit molecules with suitable pharmacological activity are identified and modified to improve potency and reduce undesired effects. Following this, the hit-to-lead discovery phase enhances the compounds' physiochemical attributes, resulting in the identification and validation of lead compounds (Kiriiri et al., 2020). Pre-clinical trials are then carried out on the drug candidate to determine the pharmacokinetics, such as drug availability, absorption, distribution, metabolism and excretion (ADME). Preliminary studies are also executed to investigate safety, such as genotoxicity, mutagenicity, general toxicology and pharmacology safety (Andrade et al., 2016). Clinical research is then carried out to identify any specific issues related to the investigational drug. Clinical trials involve phase I, Phase II and phase III trials (Lansdowne, 2022).

Clinical pharmacology is used to explain and predict the effects of drugs in humans, this expertise is also needed in several fields, including pharmaceutical companies for the development of new therapeutics, and in universities to teach health care professionals the Effective, Rational, Adjusted, Safe and Monitored Use (ERASMUS) of medicines (Buclin et al., 2012). The efficacy, safety, dosing and tolerability of the candidate drug is then determined through a series of clinical trials (Hughes et al., 2011). However, for a drug to successfully make it to market, initial testing must be conducted both *in vitro* and *in vivo* using experimental organisms.

1.1. The use of animals and alternative models in research and drug discovery

Government statistics for scientific procedures on living animals confirmed that there was a 15% decrease in the total number of procedures involving animals in 2020 compared with 2019 (Home Office, 2020). This was the lowest number of animal procedures seen since 2004. In 2020, 92% of procedures were carried out on mice, rats and fish, which have been the most used species in animal research for over a decade (Home Office, 2021).

Between 2010 and 2020 there has been a 41% decrease in animals used for basic research and a 62% decrease in animals used in applied/translational research, as shown in Figure 1.2. (Home Office, 2010-2020).

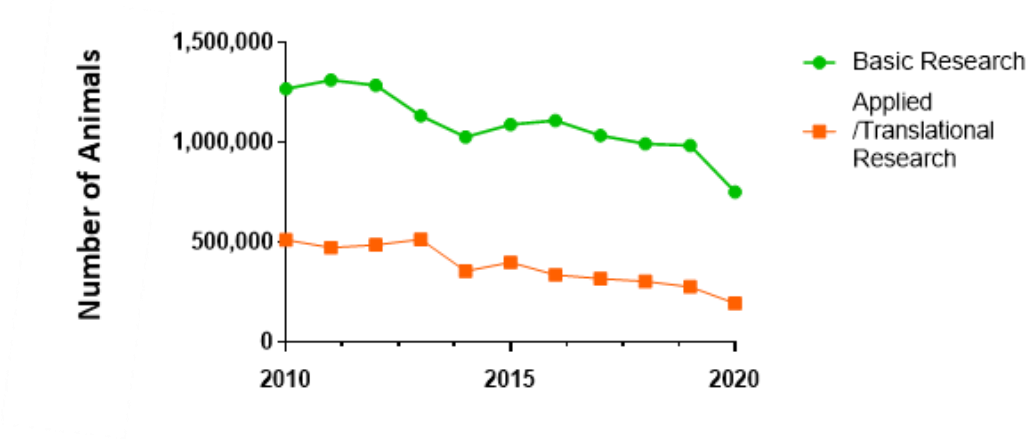

Figure 1.2. Total number of animals used each year for basic research and applied/translational research every year over 10 years. Total number of animals used in basic and applied/translation research between 2010 and 2020 (Data taken from: Home Office, 2010-2020).

Many pharmacological studies carried out in animal models are designed to indicate the bioavailability, efficacy and safety of drugs. However, animal trials are still an early phase in the development of drugs, and between 40-80% of compounds used in drug development studies are stopped from reaching further trials due to safety concerns (Cassar et al., 2020), such as predicted side effects and toxicology issues (Hartung, 2013). Despite past achievements in drug discovery and medicine, research involving animals must be designed and conducted to a suitable standard for it to be beneficial (Denayer et al., 2014) and any laboratory carrying out pre-clinical studies must comply with good laboratory practice (GLP) (Medicines and Healthcare Products Regulatory Agency, 2021).

Despite the progress made in medicine and pharmacology due to using animal models, the last decade has seen an advancement in *in vitro* systems (Clift & Doak, 2021). *In vitro* derives from the Latin "in glass", used to describe tests and experiments that are executed outside of a living organism (Eske, 2020). Any advancement and development in *in vitro* systems and procedures will enable us to better follow the principles to replace, reduce and refine animal models (Clift & Doak, 2021).

In vitro techniques include primary cell cultures, established cell lines, stem cells, organ cultures and tissue slices (Badyal & Desai, 2014). One example of a successful *in vitro* technique is the immortalisation of human-derived cells, executed by George Otto Grey in 1951 (Fabbrizi et al., 2014). Cells were derived from Henrietta Lacks, a patient with cervical cancer, now known as the HeLa cell line (Khan, 2011). Over the years this cell line has contributed greatly to developing the polio vaccine, cancer research methods and human papilloma virus (HPV) vaccines, and mapping the human genome (Samuel, 2017). More recent years have seen a development in human organ cultures, with a rise in 3D *in vitro* models that better mimic human physiology and reduce the number of animals needed in research (Al-Lamki et al., 2017). The advancements in 3D organ cultures have allowed scientists to replace the use of mice in the early screening of anti-cancer agents (Graham & Prescott, 2015).

Another technique that may reduce the number of animals in research is *in silico*, referring to computational-based techniques (Badyal & Desai, 2014), or experimentation performed by computer (Ekins et al., 2007). Using computers, the molecular structure of drugs to target specific receptors can be constructed (Arora et al., 2011). In 1995 the protease inhibitor, saquinavir, was approved for the treatment of HIV (Weber et al., 2021) and, due to the urgent need for treatment, this was designed by computer and tested in human tissue cultures and computer models, eliminating pre-clinical animal testing (Arora et al., 2011). By creating mathematical models of known human reactions, "virtual humans" have been created, that can be used to study drug absorption and evaluate the toxicity of drugs (Badyal & Desai, 2014) with recent *in silico* research capable of modelling pharmacokinetics for drugs (Kato et al., 2019).

Using methods such as these may reduce the number of animals used in research and drug development, however, they are unable to capture the complex systems of the human body and its internal environment. As such, *in vitro* studies are inevitably followed by *in vivo* studies (Uttekar, 2021). While there is a number of significant advantages when using *in vitro* systems (Moore, 2021), for example, cells derived from animals have an infinite life span, are relatively inexpensive and the results can be rapid (Uttekar, 2021), *in vitro* studies also have limitations. One of the major disadvantages is that *in vitro* systems are unable to model how drug compounds may interact with other molecules and cell types that are present in complex organs (Moore, 2021). Both *in vitro* and *in*

vivo have advantages and disadvantages. However, they are both necessary in understanding the pharmacokinetic and pharmacodynamic profile of drugs (Eske, 2020).

1.2. In vivo

In vivo, coming from the Latin term for "within the living" refers to all testing and experimentation carried out on whole living organisms, for example, testing on humans and laboratory animals (Eske, 2020). The main types of *in vivo* testing are pre-clinical animal testing followed by human clinical trials. When a new drug is developed, it is deemed unethical by society to use the drug on humans first without testing, as it may be harmful (National Academies of Sciences, Engineering, and Medicine, 1991). To avoid harm, the drug is firstly tested in animal models to make sure it is safe and effective before use in humans (National Academies of Sciences, Engineering, and Medicine, 1991). These stages provide beneficial information regarding disease progression, or how certain drugs and substances can affect an entire living organism (Eske, 2020). Animals in research have played a pivotal role in the advancement of medical science, allowing us to improve our knowledge about the diagnosis and treatment of diseases in human medicine (Graham & Prescott, 2015). Animals used in research worldwide due to their phylogenetic resemblance to humans include *Drosophila* (fruit fly), *Danio rerio* (zebrafish), *Caenorhabditis elegans* (nematode worm), *Xenopus* (frogs) and mammals such as, non-human primates, mice, rabbits, rats, cats, dogs and pigs (Mukherjee et al., 2022). Some of these species are also popular in drug discovery and development research, for example, dogs and humans have genetic similarities that extend to cancerous tumours, making dogs suitable candidates for clinical trials (Regan et al., 2022). Regan et al (2022) conducted a trial that showed the blood pressure medication, losartan, used in combination with toceranib, was able to stabilise and shrink the tumours in half of the dogs treated for osteosarcoma. Also, zebrafish represent a route to the identification and validation of novel drug targets, as genetic and morpholino oligonucleotide screens in this organism are an efficient way of assessing the roles of individual genes in disease processes (Zon & Peterson, 2005). As non-human primates closely resemble the biological make-up of humans, they have contributed to the development of the COVID-19 vaccine and Ebola vaccine, as well as playing a significant role in research into treatment of AIDS and Parkinson's disease (European Animal Research Association, 2022). However most studies that involve primates are toxicity trials that are conducted to evaluate the safety of a new drug before it is tested on humans (European Animal Research Association, 2022).

Every year millions of animals are used for basic applied/translational, forensic, and environmental research (Home Office, 2020). In vivo models used in research include rodents, non-human primates, and *Danio rerio*, or the zebrafish. However, in the United Kingdom, the use of these animals is tightly

regulated by ASPA. This act regulates all procedures that are carried out on 'protected animals' for scientific research and testing that is likely to cause pain, suffering distress or lasting harm. ASPA aims to protect all living vertebrates and any living cephalopod. Alongside this, ASPA also regulates the breeding of animals for the use of their organs or tissues in research or procedures. All animals protected by ASPA require licensing to be used in research and all scientific and medical research carried out on them in the UK must be recorded each year (Home Office, 2014).

Rodents such as mice and rats have also been a great contribution to biomedical science research. This is due to their anatomical, physiological and genetic similarity to humans, which has deemed them the preferred animal model for research into human disease and often the species of choice for pre-clinical trials (Bryda, 2013). With work dating back to before 1850, *Rattus norvegicus* was the first mammal domesticated for scientific research (Jacob, 1999). With nearly 500,000 research articles publicizing the use of rats in their work since 1966, the common laboratory rat has become an important model in the study of behaviour, biochemistry, neurobiology, physiology and pharmacology (Jacob, 1999). Although some work suggests that the rat more accurately mirrors human physiology (Hogan et al., 2013), *Mus musculus*, or the mouse, is also a long-serving model of human biology and disease, with genomic studies showing the impressive genetic homologies between mice and humans (Perlman, 2016). Research involving mice has had a considerable impact on our knowledge of the adaptive immune system, as mouse research led to the discovery of the major histocompatibility complex genes and the T cell receptor, which resulted in our understanding of antibody synthesis (Khanna & Burrows, 2011). Mice are also the most frequently used models in drug discovery and development, and with the unveiling of genetically engineered models (GEM) in the late 20th century, the use of mice in research has progressed allowing them to become a more effective *in vivo* model than ever before (Vitale, 2019). GEM mice are often used in drug development to determine target validation or as a specific animal model of human disease, and since the 1980's several different types of GEM mice have been used in pre-clinical trials, including transgenic, knockout, and knock-in models (Lee, 2014). Traditional and modern mouse genome editing tools, along with the addition of genetic diversity in more recent modelling systems, go hand in hand to improve the mouse model in biomedical research and improve the potential for pre-clinical drug discovery and personalised medicine (Zuberi & Lutz, 2016).

The use of non-human primates in research has an essential role in basic and translational biomedical research (Capitanio & Emborg, 2008). The variety of non-human primates used in research can be split into New World species, which include marmosets, and Old World species, which include macaques, such as the long-tailed or rhesus macaque (Chatfield & Morton, 2018). Chimpanzees have also been used in research, as they are genetically and structurally like humans, with more than 98%

Deoxyribonucleic acid (DNA) homology (Didier et al., 2016). As non-human primates have genetic similarities to humans, they are highly valued in research and are extremely beneficial for testing the safety of new drugs and studying infectious diseases (Chatfield & Morton, 2018). Alongside this, non-human primates are useful for neurophysiology research, as they can be trained to respond to stimuli and their central nervous system (CNS) responses can be monitored (Cyranoski, 2016).

Since its introduction as an experimental *in vivo* model in the 1980s zebrafish have become an invaluable asset in developmental biology research (Veldman & Lin, 2008). Zebrafish possess genomic and molecular similarities to humans and other vertebrates (Veldman & Lin, 2008), and are considered appropriate models to investigate development, genetics, immunity, behaviour, physiology and nutrition (Teame et al., 2019). These tropical freshwater fish have an advantage over mammalian vertebrate models, such as rodents, as the rapid process of zebrafish embryo development is external and can be visually observed (Veldman & Lin, 2008). Previous studies have shown that zebrafish contribute to the greater understanding of biological activities of orthologs to human disease-related genes. When a direct comparison of zebrafish and human protein-coding genes was carried out, it revealed that 71.4% of human genes have at least one zebrafish ortholog (Vilella et al., 2009), and 69% of zebrafish genes have at least one human ortholog (Howe et al., 2013). Despite playing a key role in genetic research, zebrafish have also been used to study infectious disease, cancer, cardiovascular disease, diabetes, muscle disorders, and haematopoiesis (Teame et al., 2019)

While experiments carried out on vertebrates are regulated in most countries, those on invertebrates are not, which has led to inaccurate statistics regarding the use of invertebrates in research (Badyal & Desai, 2014). Invertebrates represent a diverse group of animals that make up more than 95% of the overall species on Earth (Crespi-Abril & Rubilar, 2021). They are often used for research and teaching as they have several advantages over vertebrate models (Crespi-Abril & Rubilar, 2021), such as easier and less expensive to culture a maintain, simpler organisms and shorter life cycle (Smith et al., 2011). Two of the most common invertebrates used in research include *Caenorhabditis elegans*, a nematode and one of the main model species in life sciences (Zhang et al., 2017) and *Drosophila melanogaster*, more commonly known as the fruit fly and its contribution to genetics (Tolwinski, 2017).

Unlike other invertebrates the diverse group of invertebrate species that are cephalopods are protected by ASPA, meaning that the appropriate authority and licensing is needed to carry out any procedures on them (Home Office, 2014). Cephalopods are often termed “advanced invertebrate” and have been utilised in neuroscience research for more than a century, mainly due to their complex and centralised nervous system (Fiorito et al., 2014). One of the greatest success stories in biology was

in 1939, when Cole and Curtis demonstrated that action potential is due to a significant surge in membrane conductance (Cole & Curtis, 1939), following on from this Hodgkin and Huxley observed and recorded the first intracellular action potential from squid axon (Hodgkin & Huxley, 1939). They demonstrated that depolarisation could be separated into two different components, a rapid inward current of Na^+ ions followed by a slower outward current of K^+ ions (Hodgkin & Huxley, 1952). Since these findings, Hodgkin and Huxley's theory of the action potential has become one of the great success stories in biology (Schwiening, 2012).

Despite these animal models being invaluable contributions to the advancement of medicine and pharmacology, not one can be a complete model for human disease due to differences in biological structures and functions compared with humans. Although they may give us beneficial insights into the effects of drugs on biological systems, we cannot fully replicate this in a human model and expect to see the same outcome. This means there is a call for an *in vivo* model that would potentially reduce the number of animals protected by ASPA being used in research.

1.3. The use of alternative *in vivo* models

The use of animals for research and medicine has been a topic of heated debate for many years in the UK (Festing & Wilkinson, 2007). According to the classic definition given by Russel & Burch, the term 'alternative' refers to any technique that replaces the need for animals in a certain assay or refines an existing technique in order to reduce the amount of animal suffering in research (Russel & Burch, 1960). This framework was first published in 1959 by Russell and Burch to ensure more humane research and the principles have been put in place to replace, refine and reduce the number of animals used in research (3R's) (Tannenbaum & Bennett, 2015).

- To replace would be to completely replace or avoid methods using animals in research.
- To refine would be to minimise animal suffering and improve their welfare.
- To reduce would be to reduce the number of animals used per experiment.

To replace would be to use alternative model systems such as *in silico* or *in vitro*. However by utilising invertebrate models such as *Drosophila*, and *C. elegans* we are able to reduce the number of procedures involving vertebrate animal models (Wilson-Sanders, 2011).

Drosophila genome is 60% homologous to that of humans and around 75% of the genes accountable for human diseases have homologs in fruit flies (Ugar et al., 2016). For over a century *Drosophila* has been used to study many biological processes, including inheritance, embryonic development, learning, behaviour and ageing (Jennings, 2011). The classically trained embryologist, Thomas Hunt Morgan, chose to use the fruit fly as a model organism in 1909 (Letsou & Bohmann, 2005) since then this low-cost and easily cultured model has earned scientists six Nobel prizes (Dutchen, 2018). *Drosophila* is a valuable model in cancer research as they offer the opportunity to study the regulation of cell death in response to different stimuli, and the role of cell death in normal development, tissue homeostasis and in a range of disease models (Steller, 2008). The use of this organism has provided invaluable information, such as within oncology. In cancer apoptosis is not triggered, aiding the process by which tumours spread. However, as a result of research involving *Drosophila*, it has been established that inhibitors of apoptosis proteins (IAPs) act to block cell death, resulting in the spread of cancer due to the lack of apoptosis. As a result of these findings, drugs are now being tested that inhibit IAPs in humans, which will allow apoptosis to regain control of cells (McKie, 2017). More recent work by Mackay & Anholt at Clemson University Centre for Human Genetics has revealed the invaluable use of invertebrates in the study of drugs of abuse. In *Drosophila*, specific cell clusters in the brain are affected by acute cocaine exposure (Clemson News, 2021) and by creating an atlas of cocaine-modulated gene expression changes in the fly brain, they found that transcriptional changes in response to acute cocaine consumption are rapid, widespread in neurons and glia, and affects male

and female *Drosophila* differently (Baker et al., 2021). This could be a potential basis for the development of drugs to treat or prevent addiction in humans (Clemson News, 2021). *Drosophila* is a valuable model organism for use in the clinical drug discovery process (Bell et al., 2009), as novel drugs can be tested on them much more quickly than in mammalian models. *Drosophila* can be used to observe the effects of novel drugs on the biochemical processes within humans that control fundamental cellular activities such as cell division, differentiation and movement (Jennings, 2011). Using *Drosophila* in research has several advantages over using vertebrate models, including the cost to culture and maintain in laboratory conditions, their short life cycle and ability to produce large numbers of externally laid embryos, and the numerous ways they can be genetically modified (Jennings, 2011).

Other invertebrates have also provided extensive knowledge to medical research, helping to further understand the underlying biology of humans. In 1974 Brenner published a manuscript entitled 'The genetics of *Caenorhabditis elegans* (Brenner, 1974), and since then the small nematode roundworm has become one of the fundamental model organisms for molecular and cell biology (Nigon & Félix, 2018). Under suitable conditions, *C. elegans* have a life cycle of 3.5 days and a lifespan of around 2-3 weeks (Markaki & Tavernarakis, 2010). They are cultured in large numbers and adults tend to be around 1mm in length and feed on bacteria such as *Escherichia coli* on agar plates or in a liquid medium (Markaki & Tavernarakis, 2010). *C. elegans* have several advantages over vertebrate models in biomedical research, for example, they can rapidly reproduce, can be easily cultured in laboratory conditions and are inexpensive to maintain (Wilson-Sanders, 2011). Other advantages include *C. elegans* animal-like physiological properties and its ability to replicate human diseases (Yokoyama, 2020). Not only have *C. elegans* been used as a model organism to study mitochondrial diseases, Parkinson's disease and the immune system (Yokoyama, 2020), but they have also been useful for studying the ageing process, as they undergo several different phases of life which can be genetically and physiologically observed (Zhang et al., 2020), and studies have shown that a substantial variety of compounds, such as minocycline, metformin and resveratrol, can extend *C. elegans* lifespan (Berkel & Cacan, 2021). Another major beneficial attribute that *C. elegans* offer is the ability to execute genetic analysis. *C. elegans* were the first multicellular organism to have its entire genome sequenced and published (*C. elegans* Sequencing Consortium, 1998). Although small in size, *C. elegans* possess organs systems that are present in more complex organisms, such as a nervous system that is made up of 302 neurons (Bono & Villu Maricq, 2005), digestive system, reproductive system and musculature (Apfeld & Alper, 2018). Initially, *C. elegans* behaviour seemed to be limited to basic processes, such as feeding, egg-laying and locomotor (Brenner, 1974), however over the years research has shown that *C. elegans* possess a multitude of behaviours that can be measured (Meneely et al., 2019). As in humans, the

neuronal networks in *C. elegans* control their movement, as their locomotion is controlled by excitatory cholinergic and inhibitory GABAergic motor neurons located in the ventral nerve cord of the worm (Chalfie et al., 1985). Strong evidence shows that there are seven neurotransmitters present and functioning in the neurons of *C. elegans*: acetylcholine, dopamine, tyramine, octopamine, Gama-aminobutyric acid (GABA) and glutamate (Loer & Rand, 2010). The fairly short lifespan of *C. elegans*, along with the availability of genetically modified nematode strains and the simplicity of modulating genes by ribonucleic acid interference (RNAi) has made them an increasingly important and beneficial model in pharmacological and toxicological sciences (Koch et al., 2014). A study by Spensley et al (2018) looked at the acute effect of drugs on *C. elegans* movement by exposing the organism to aldicarb and cyanide. This demonstrates that nearly half of all the compounds that affected *C. elegans* locomotion show the same response, movement is rapidly inhibited causing an early phase of near-paralysis, which *C. elegans* later recover from enabling them to regain movement. This study also revealed that their response to the same drug can be different depending on the worm's different development stages and different drug doses (Spensley et al., 2018). Research designed to rank toxicity in *C. elegans* has also shown promising results, as they have often shown comparable results to rodent oral lethal dose (LD_{50}) ranking (Hunt, 2017). For example, an early ranking study that used *C. elegans* maintained on plates with test articles dissolved in agar found that the toxicity order for eight metal salts based on *C. elegans* adult mortality coincided with rodents, such as rats and mice, oral LD_{50} ranking, and cost one-tenth of rodent testing (Williams & Dusenbery, 1988).

The number of published studies using non-mammalian species has significantly increased since the 1990s by 90.9%, of which 68.4% used *Drosophila* and *C. elegans* (Freires et al., 2017). These species have provided revolutionary biological insights into various areas including neurobiology, genetics, reproduction, development, cell death, innate immunity and ageing (Castillo & de la Guardia, 2017). More established invertebrate models could be beneficial to the process of drug discovery, allowing for effective High-Throughput Screening and technological development. Alongside this, invertebrates have powerful genomics which allows for the identification of potential drugs and the characterisation of drug targets (Castillo & de la Guardia, 2017). With species such as *Drosophila* and *C. elegans* being beneficial within the medical research field, it would make sense, from an ethical point of view, to consider other invertebrate species for use as *in vivo* models.

1.4. *Lumbriculus variegatus* as an alternative *in vivo* model

While the research community must commit to promoting and developing alternatives to *in vivo* models (Fontana et al., 2021), there is still a need to utilise animal models to develop new, improved treatments, and keep up with newly discovered diseases. Biomedical research involving animals has been essential for medical progress, and although ethical concerns about the use of vertebrate animals have resulted in a decrease in animals used in both research and education, invertebrate models can assist in the replacement of vertebrate models (Wilson-Sanders, 2011).

Between 2010 and 2020 there has been a 79% decrease in animals used for training and education purposes (Figure 1.3.). The number of animals used for training and education decreased 88% between 2001 and 2020. Although these statistics demonstrate that the science community is supporting the movement toward replacing, reducing and refining (Clift & Doak, 2021), The Association of the British Pharmaceutical Industry (ABPI) has published several reports, from 2005 to 2018, which highlighted the skills shortage of UK graduate, postgraduate and post-doctoral *in vivo* pharmacologists (Home Office, 2020) and so training scientists must receive alternative upskilling in *in vivo* pharmacology to address this skills gap.

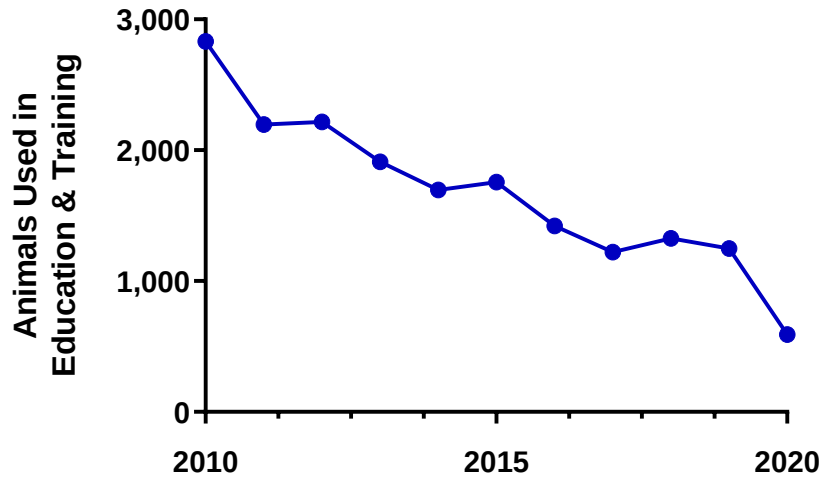

Figure 1.3. Total numbers of animals used each year for educational and training purposes over a decade. The decrease in total number of animals used in education and training between 2010 and 2020. (Adapted from: Home Office, 2010-2020).

The current study utilised freshwater oligochaete species *Lumbriculus variegatus* (Figure 1.4.), more commonly known as the blackworm, as a novel *in vivo* model for pharmacology testing. *L. variegatus* are inexpensive and easy to maintain and culture, making them a suitable model for research purposes. *L. variegatus* range from 2 to 8 cm (Seeley et al., 2021), meaning they are large enough to observe without the need for specialist equipment. Developing *L. variegatus* as an *in vivo* model will also adhere to the 3R's principles of Replacement, Refinement and Reduction of animal models in research.

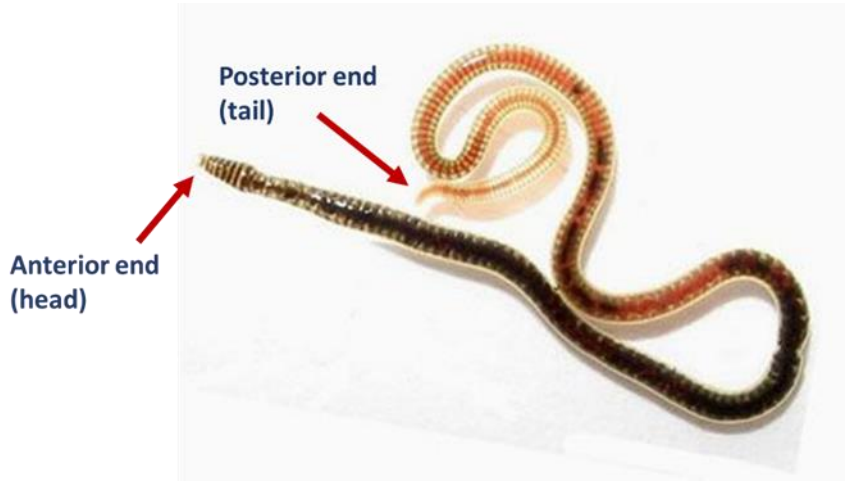

Figure 1.4. Image of Lumbriculus variegatus with anterior and posterior end labelled.

Although *L. variegatus* are not widely used in research by biomedical scientists, they can exhibit a range of biological phenomena including swimming reflexes, blood pulsation, giant nerve fibre action potentials and sublethal sensitivity to pharmacological substances (Drewes, 2004). Despite not being widely used for biomedical research, *L. variegatus* has been utilised for environmental toxicology studies, as they are found in marshes and lakes, meaning they are likely to be exposed to environmental toxicants and pollution (Drewes, 1997). This was demonstrated by O'Gara et al. (2004) in a study that showed that the toxic effects of copper in the environment have sub-lethal effects on *L. variegatus* behaviours. A study by Ding et al. (2001) also observed *L. variegatus* behaviour when exposed to ivermectin, the popular anti-parasitic drug used in cattle that is often excreted into the environment by domestic animals that have been receiving ivermectin treatment. Ding et al. (2001) demonstrated that ivermectin inhibited swimming, reversal, and crawling frequency and speed in *L. variegatus* (Ding et al., 2001).

L. variegatus are detritus feeders and so contribute to the breakdown of organic materials as well as serving as nutriment for larger animals (O'Gara et al., 2004). As *L. variegatus* are a prey species, they

possess rapid withdrawal behaviours. When in water they place themselves with their anterior burrowed into sediment and their posterior extended up towards the water surface, this allows them to respond with rapid withdrawal to stimuli or shadow (Ding et al., 2001). Alongside this, *L. variegatus* exhibit other locomotor behaviours such as crawling, body reversal and helical swimming (O'Gara et al., 2004).

L. variegatus breathe via cutaneous respiration and obtain oxygen through the dorsal surface of their tail (Drewes, 2004), another reason they place themselves in sediment with their tails facing toward the water surface (Bohrer, 2006). Like many annelids, *L. variegatus* have a closed circulatory system (Alvarez-Collazo et al., 2014), that is made up of a dorsal blood vessel (DBV) and ventral blood vessel (VBV) (Drewes, 2004). *L. variegatus* blood is red due to a haemoglobin-like pigment known as erythrocruorin that is present in the blood plasma (Jamieson, 1981). Blood flows from the posterior end of *L. variegatus* to the anterior end via the DBV, this is then carried back to the posterior end via the VBV (Drewes, 2004). As the body wall of *L. variegatus* is transparent, the pulsation of the DBV can be observed using light microscopy (Lesiuk & Drewes, 2001).

Annelids have a plasma coagulation process that is mechanistically similar to mammals. In mammals, plasma coagulation converts soluble fibrinogen into insoluble fibrin, much like this, the annelid clotting pathway involves fetidin which is also converted from a soluble form to an insoluble form (Kwong et al., 2016). *L. variegatus* possess thrombin-like proteases that cleave fetidin to initiate the coagulative process, which is analogous to the process of coagulation in humans (Tweeten & Reiner, 2012).

L. variegatus central nervous system is comprised of a cerebral ganglion and a ventral nerve cord that extends throughout the length of the worm (Figure 1.6). The ventral nerve consists of sensory neurons, motor neurons and interneurons, many of these neurons extend into the "neuropile", which is found at the centre of the ventral nerve cord. This creates connections that provide a biological basis for both neuronal circuits and reflex systems that manage *L. variegatus* movements and behaviour (Drewes, 2002).

The ventral nerve cord consists of giant nerve fibres, termed the medial giant fibre (MGF) and the lateral giant fibre (LGF) (Drewes, 2002). These giant nerve fibres play a role in *L. variegatus* ability to perform withdrawal behaviours that are evoked by shadows or stimuli. When the anterior end of *L. variegatus* is stimulated it initiates excitation of the MGF system, whereas stimulation of the posterior end excites the LGF system (Drewes, 1997). A study by Lybrand et al (2019) suggests that MGF-mediated escape responses in *L. variegatus* are glutamatergic. They came to this conclusion by exposing *L. variegatus* to glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dion, which proved to inhibit evoked MGF responses (Lybrand et al., 2019). Both the MGF system and LGF system

trigger activity in both the motor neurons and longitudinal muscle, causing the length of *L. variegatus* body to shrink (Drewes, 2004). A study by Drewes (1997) shows that both MGF and LGF spiking that causes withdrawal responses will not occur, unless evoked, in unintoxicated *L. variegatus*. Tactile stimulation of the anterior of *L. variegatus* causes body reversal (Figure 1.5 A), and stimulation of the posterior elicits helical swimming (Figure 1.5 B), where the worms corkscrew through the water (O'Gara et al., 2004).

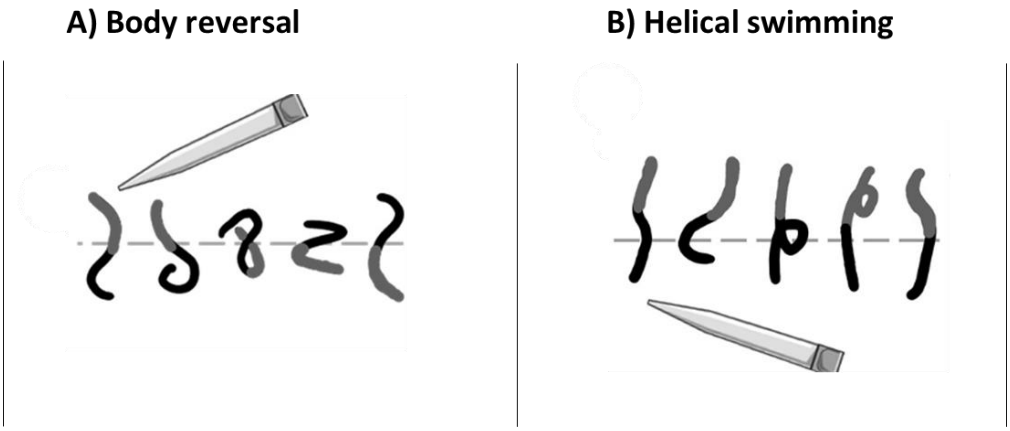

Figure 1.5. Diagram of *L. variegatus* stereotypical behaviours. Stimulation of the anterior end causes worm to perform body reversal (A), and stimulation of the posterior end causes helical swimming movement (B). (Adapted from Seeley et al., 2021)

These behaviours have previously been described and used to determine the effects of exogenous compounds on *L. variegatus* (Seeley et al., 2021), and are easily quantifiable without the need for specialist equipment. As *L. variegatus* locomotor behaviours are highly stereotyped, it makes them an ideal model organism for pharmacology and sublethal toxicology testing (Ding et al., 2001). A study by Kwong et al (2016) also looked at the coagulative properties in *L. variegatus*. As *L. variegatus* are low-hazard models, this allows their healing mechanisms to be widely researched, especially as their average coagulation time is short and can be observed within the lab in one session using a low-resolution stereomicroscope (Kwong et al., 2016).

One of the most current areas of focus for invertebrate research is drug discovery and development. There may also be potential for an invertebrate role in toxicity and efficacy testing of new pharmaceuticals, abolishing the need for preliminary vertebrate testing (Wilson-Sanders, 2011). Although *L. variegatus* behaviours have previously been described and used as observations in ecological toxicology studies, little is known about their reaction to drug compounds (Seeley et al.,

2011), or what mechanism is used. As shown in previous studies, drugs and other compounds can quickly diffuse through *L. variegatus* surface (Ryan & Elwess, 2017), meaning there is less need for specialist equipment when carrying out any pharmacological and toxicological studies.

Although previous studies have reviewed the effects of drugs, such as fluoxetine, carbamazepine, and diclofenac (Karlsson et al., 2016; Nentwig, 2007; Oetken et al., 2005), further investigation is required to determine the adequacy of *L. variegatus* as an *in vivo* model. To further characterise *L. variegatus* pharmacological responses we exposed them to several drug compounds, including exogenously administered chemicals that other models endogenously produce, such as neurotransmitters and measured the effects.

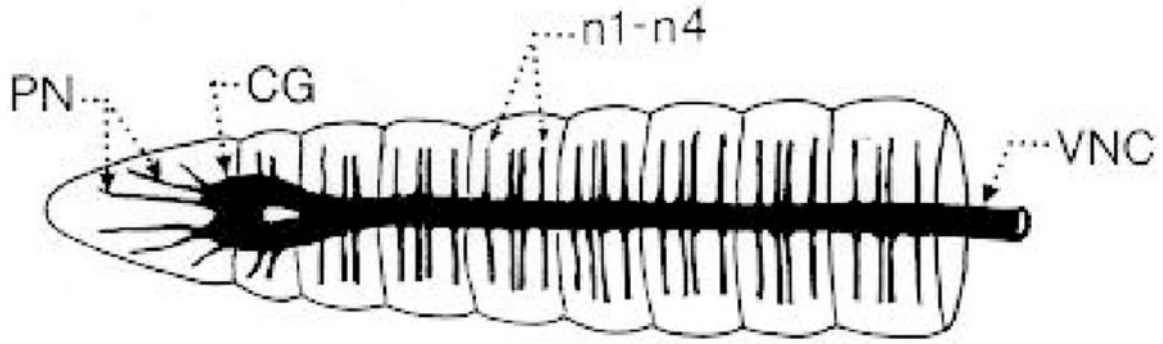

Figure 1.6. Dorsal view of the central nervous system in the anterior end of *L. variegatus.* Abbreviations: PN – prostomial nerves, CG – cerebral ganglion, n1-n4 – segmental nerves 1-4, VNC – ventral nerve cord (Drewes, 2002).

Table 1.1. List of the advantages and disadvantages of *Lumbriculus variegatus* in research.

Advantages	Disadvantages
Possess stereotypical behaviours that are amendable to quantification (O'Gara et al., 2004).	*L. variegatus* reproduces primarily through asexual reproduction (Martinez et al., 2006). This means it is difficult to determine the exact age of each worms used in experimentation (Aikins et al., 2023).
Have previously been used in research for ecological toxicology testing (Nentwig, 2007; Karlsson et al., 2016).	Not a lot of existing data surrounding *L. variegatus* reaction to drug compounds (Seeley et al., 2021)
L. variegatus are a low-cost model and are exempt from many regulatory and ethical constraints that are associated with other in vivo models (Seeley et al., 2021).	To limit variation in colony, *L. variegatus* must be maintained for a minimum of three months before experimentation (Seeley et al., 2021).
Compared with *C. elegans* which are ~1mm in size, *L. variegatus* range in size from 50-80mm, making them easier to observe individually (Seeley et al., 2021).	Unlike *C. elegans*, there is little data surrounding *L. variegatus* genetic make-up and full genome is yet to be sequenced (Gustafsson et al., 2009).

1.5. Overview of drugs and compounds

We examined the effects of a range of drug compounds with diverse pharmacodynamic properties within *L. variegatus* as a proof-of-concept study in the field of pharmacology. We chose a broad range of structurally divergent compounds including lidocaine, quinine, dantrolene, dopamine, haloperidol, sulpiride, GABA, glycine, bicuculline, ethanol, and 2,4-dinitrophenol (DNP).

1.5.1. Lidocaine

Lidocaine is primarily used as a local anaesthetic and anti-arrhythmic drug, however, there is strong evidence suggesting that lidocaine infusions can be used in the treatment of hyperalgesia (Hermanns et al., 2019). Lidocaine also has a remarkable safety profile compared to older local anaesthetics (Beecham et al., 2022). Local anaesthetics such as lidocaine work by inhibiting action potentials in excitable tissues and blocking the transmission of pain impulses (Taylor & McLeod, 2020).

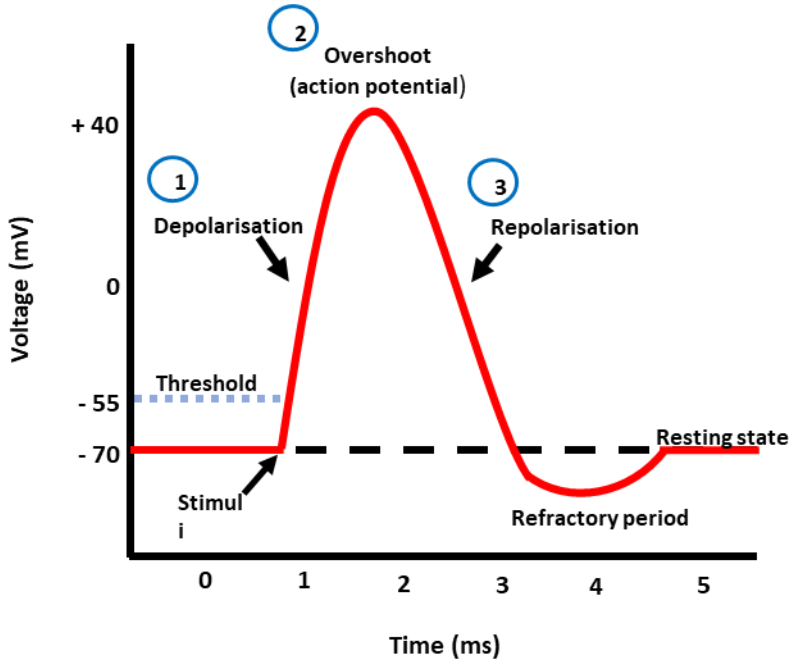

Figure 1.7. Diagram showing action potential curve and phases (Adapted from Khanna, 2021). Action potentials are signals generated and conducted within neurons to transmit signals to target tissues (Vaskovic, 2022). These signals consist of three phases: depolarisation, overshoot and repolarisation (Vaskovic, 2022).

When a cell is at resting membrane potential, the concentration gradient is controlled and maintained by sodium/potassium ATPase via active transport (Figure 1.8 A) (Grider et al., 2022). An action potential is generated when the membrane potential is altered to that of threshold potential (Vaskovic, 2022), this occurs at the axon hillock as a result of depolarisation (Khanna, 2021). Depolarisation occurs due to an electrical stimulus, this causes voltage-gated sodium ion channels to open, allowing sodium ions to flow into the intracellular space (Figure 1.8 B) (Kress & Mennerick, 2009). This influx of sodium ions further depolarises the membrane, resulting in an upstroke of the action potential (Kress & Mennerick, 2009). Once the action potential has been produced, there is a decrease in sodium permeability as a result of sodium channel closure (Vaskovic, 2022). The positive potential inside the cell opens the voltage-gated potassium channels, resulting in an efflux of potassium ions (Figure 1.8 C) and a negative membrane potential (Khanna, 2021). All action potentials are followed by a refractory period, where the sodium ion channels become inactive, and then slowly become active again (Khanna, 2021).

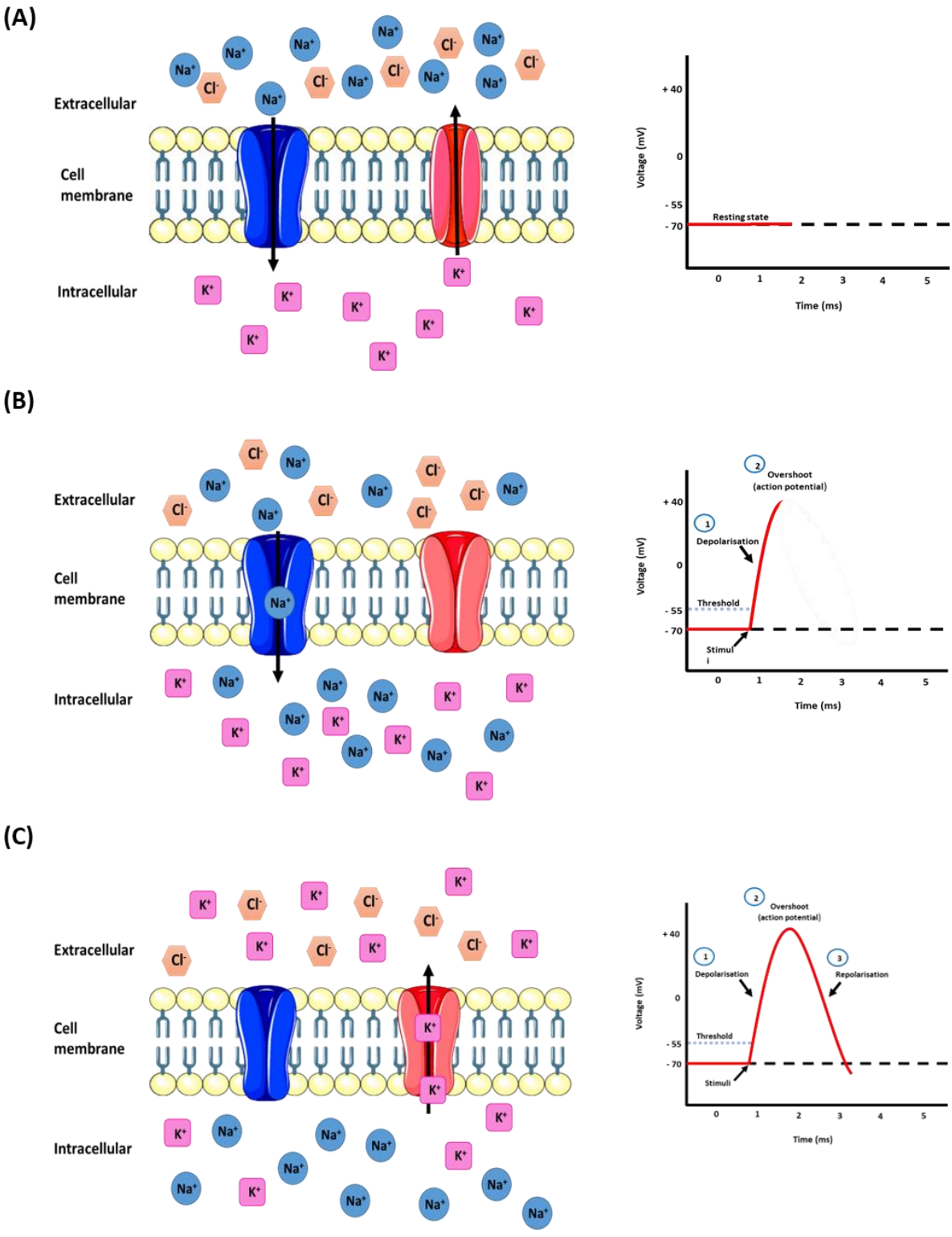

Figure 1.8. Schematic demonstrating ions involved in and concentration gradient of ions during each phase of action potential (Adapted from Khanna, 2021). The concentration gradient of a cell in resting state is controlled and maintained by sodium/potassium ATPase via active transport (Grider et al., 2022). During depolarisation the voltage-gated Na+ channels open, allowing an influx of Na+ ions (Grider et al., 2022). Voltage-gated K+ channels are then opened in order to mediate the resting potential; this is known as repolarisation. An ATP-driven pump (Na/K-ATPase) then begins the movement of Na+ ions out of the cell and K+ ions into the cell (Grider et al., 2022).

Lidocaine inhibits action potentials by binding to voltage-gated sodium ion channels, blocking the flow of ions through the membrane (Figure 1.9) (Cummins, 2007). Lidocaine deactivates voltage-gated sodium channels reducing the excitability of neurons, and reducing and preventing pain (Yang et al., 2020). It is suggested that lidocaine binds with low affinity to Na^+ channels when the channel is in a rested state and binds with high affinity to the inactivated state of the channel (Bennett et al., 1995). The half-life of lidocaine is 1.5 – 2 hours (Torp et al., 2023).

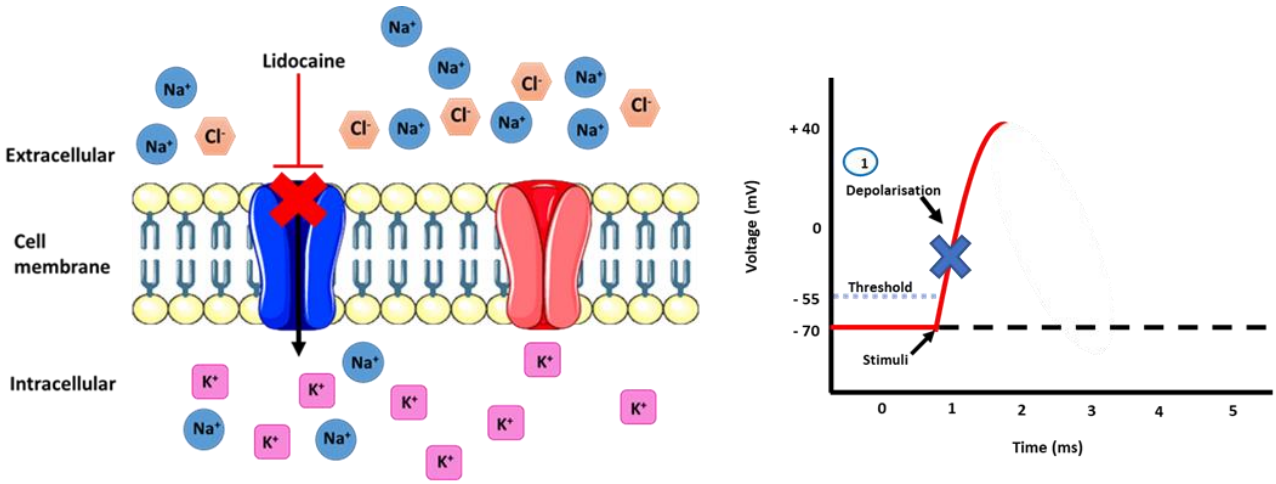

Figure 1.9. Schematic of lidocaine mechanism of action. Lidocaine blocks voltage-gated sodium receptors, resulting in inhibition of action potential (Cummins, 2007).

1.5.2. Quinine

Quinine is an alkaloid extracted from *Cinchona officinalis,* known as cinchona bark found in South America (Saguil & Lauters, 2016). It was the first successful anti-malarial drug (Uzor, 2020), and since the 1930s has been researched as a treatment for muscle cramps (Katzberg et al., 2010). Quinine is a non-selective sodium and potassium channel blocker (Lin et al., 1998), and has shown to have antiarrhythmic effects in animal models (Sheldon et al., 1995). Potassium channels have a role in the repolarisation of the membrane after an action potential, this is necessary for terminating the action potential signal and returning the membrane to a negative resting potential (Kim & Nimigean, 2016). When potassium channels are blocked, this delays repolarisation, leading to an increase in action potential duration and an increase in the effective refractory period (Figure 1.10) (Klabunde, 2011). The half-life of quinine is 11 – 18 hours (Achan et al., 2011).

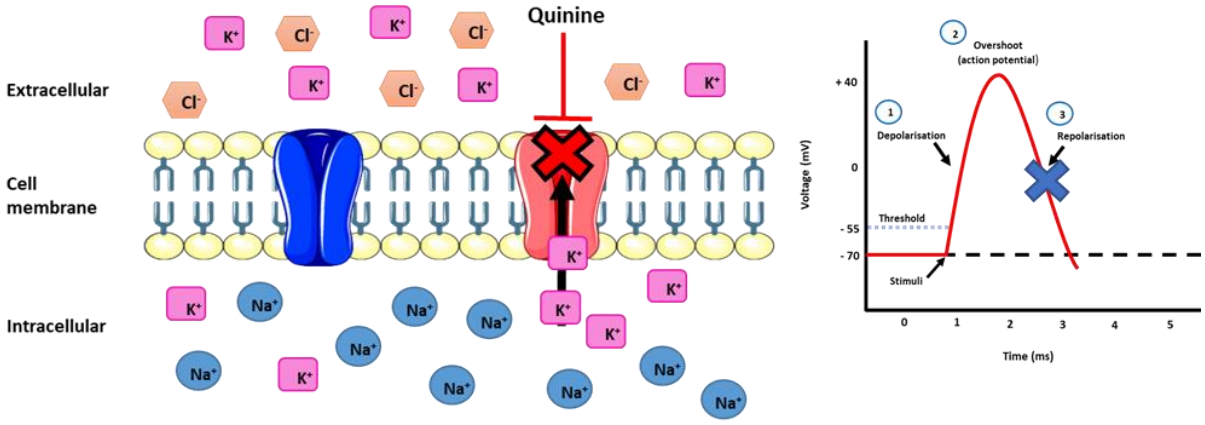

Figure 1.10. Schematic of quinine mechanism of action on potassium channels, resulting in a delay in repolarisation of action potential.

1.5.3. Dantrolene

Dantrolene is a postsynaptic muscle relaxant (Ratto & Joyner, 2022). It works by mediating ryanodine receptor calcium release**,** located in the membrane of the sarcoplasmic reticulum in muscle cells and the endoplasmic reticulum in neurons (Wang et al., 2020). Dantrolene is the only effective treatment for malignant hyperthermia, a fatal condition induced by inhaled anaesthetics and succinylcholine (Shi et al., 2019). Malignant hyperthermia is a rare pharmacogenetic disorder of the skeletal muscle that presents as a hypermetabolic response, in which an uncontrollable increase in oxidative metabolism in skeletal muscles saturates the body's capacity to supply oxygen and regulate body temperature (Kaur et al., 2019). Dantrolene is also neuroprotective in neurodegenerative diseases, such as Alzheimer's disease (Shi et al., 2019), cerebral ischaemia, Huntington's disease, amyotrophic lateral sclerosis and trauma (Wang et al., 2020).

Although invertebrate nervous systems are less complex than vertebrates, both evolved from common ancestors that possessed neurons and structured central nervous systems (Arendt et al., 2008). Currently, there is not enough information surrounding *L. variegatus* neurophysiology for us to fully understand the neuropharmacological mechanisms in which drugs exert their effects. This means we are unable to compare mechanisms of action between species. Dantrolene has a high affinity activating and lower affinity inhibiting the RYR1 binding site on the sarcoplasmic reticulum membrane (Nelson et al., 1996). The half-life of dantrolene is 4 – 8 hours (Ratto & Joyner, 2023).

1.5.4. Dopamine

Dopamine is an endogenous monoamine catecholamine (Sotnikova et al., 2010) that acts on both the central nervous system (CNS) and peripheral nervous system (PNS) (Belkacemi & Darmani, 2020). It has an essential role in the regulation of reward, motivation and movement (Kim et al., 2017), however, the activity of dopamine can be influenced in different ways depending on factors such as unpredictable rewards, drug exposure or stress (Di Giovanni, 2010). Dopaminergic neurons also play a significant role in abnormal brain functions and processes (Marsden, 2006), for example, the debilitating neurodevelopmental disorder, schizophrenia, is characterised by dysregulation of dopaminergic and glutaminergic neural systems (Rajasekaran et al., 2015) and there is significant evidence that the dopamine system is hyperresponsive in schizophrenia (Grace, 2016). Dopamine also plays a key role in drug addiction, depression, Parkinson's disease and ADHD (Marsden, 2006). Alongside this, dopamine receptors are involved in hormonal regulation and have an influence on the immune system (Beaulieu et al., 2015).

There are five subtypes of dopamine receptors (D_1 to D_5), all of which are well-established targets in the clinical pharmacology of various illnesses and disorders (Beaulieu et al., 2015). Dopamine receptors are part of the G-protein coupled receptor (GPCRs) superfamily and are classified as either D_1-class receptors (D_1 and D_5) or D_2-class receptors (D_2, D_3 and D_4) (Spano et al., 1978). D_1-class receptors are generally coupled to $G\alpha_{s/olf}$ proteins and initiate the activity of adenylyl cyclase and the production of cyclic adenosine monophosphate (cAMP), whereas D_2-class receptors inhibit the production of cAMP as they are coupled to $G\alpha_{i/o}$ proteins (Beaulieu et al., 2015). There is a difference in the affinity of dopamine depending on which receptor it is binding to. D2-like receptors have 10-100 times greater dopamine affinity than the D1-like family receptors, meaning that the D2-like vs D1-like receptor signalling can change depending on extracellular dopamine concentrations (Martel & Gatti McArthur, 2020). The half-life of dopamine is 1 – 5 minutes (Sonne et al., 2023).

1.5.5. Haloperidol

Haloperidol is a first-generation 'typical' anti-psychotic (Sattler et al, 2020), or neuroleptic (Jibson, 2020), that is highly effective when used to relieve psychotic symptoms in those with schizophrenia (Marwari & Dawe, 2019), a disorder which is associated with hyperdopaminergic transmission (Li et al, 2016).

Haloperidol is a dopamine receptor antagonist, meaning it binds to dopamine receptors and inhibits dopaminergic effects (Figure 1.11) (D. A. Mandal, 2019). Although first-generation anti-psychotics

exert their actions primarily through dopamine D_2 receptors (Li et al, 2016), haloperidol is a non-selective dopamine receptor antagonist, binding to dopamine D_1 receptors and with high-affinity to dopamine D_2 receptors (Marwari & Dawe, 2019). The half-life of haloperidol is 14 – 26 hours (Mandal, 2019).

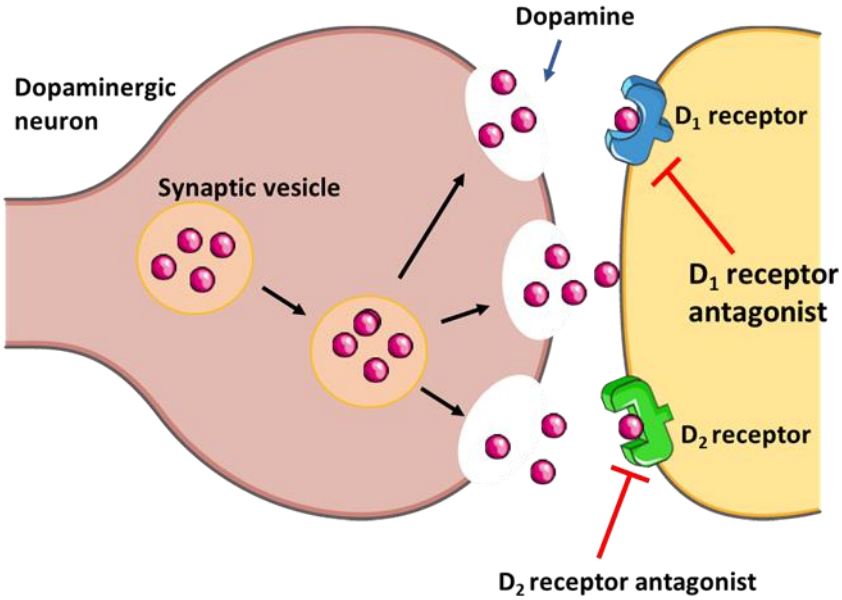

Figure 1.11. Schematic of mechanism of action of D_1 receptor and D_2 receptor antagonist. As haloperidol is a non-selective dopamine D1 and dopamine D2 receptor antagonist, it is able to bind to both receptors inhibiting dopaminergic effects (Marwari & Dawe, 2019).

1.5.6. Sulpiride

Much like haloperidol, sulpiride is also an antipsychotic used in the treatment of schizophrenia (Soares et al., 1999). Although there is a similarity between the mechanism of action of haloperidol and sulpiride, sulpiride is structurally different compared to other anti-psychotics (Wagstaff et al., 1994).

As sulpiride is an 'atypical' neuroleptic (Rich, 1984) it is used to treat namely the negative symptoms of the disorder (Soares et al., 1999). Sulpiride is a benzamine derivative (Wagstaff et al., 1994) that acts as a dopamine receptor antagonist in the brain (Jenner & Marsden, 1982), selectively inhibiting dopamine D_2 and dopamine D_3 receptors (Asad et al., 2020). The half-life of sulpiride is 8 hours (Electronic medicines compendium, 2019). Sulpiride has a high affinity for dopamine receptors that have an impact on emesis and prolactin secretion (O'Connor & Brown, 1982).

1.5.7. GABA

Gamma-aminobutyric acid (GABA) is one of the main inhibitory neurotransmitters within the human brain (Boonstra et al., 2015) and alongside glutamate and glycine, is one of many amino acid neurotransmitters within the CNS (Shah et al., 2002). The half-life of GABA is 5 hours (Li et al., 2015). GABA receptors can be divided into two types depending on their mechanism of action (Hinton & Johnston, 2018), ionotropic receptors and metabotropic receptors (see Figure 1.12) (Kannampalli & Sengupta, 2015).

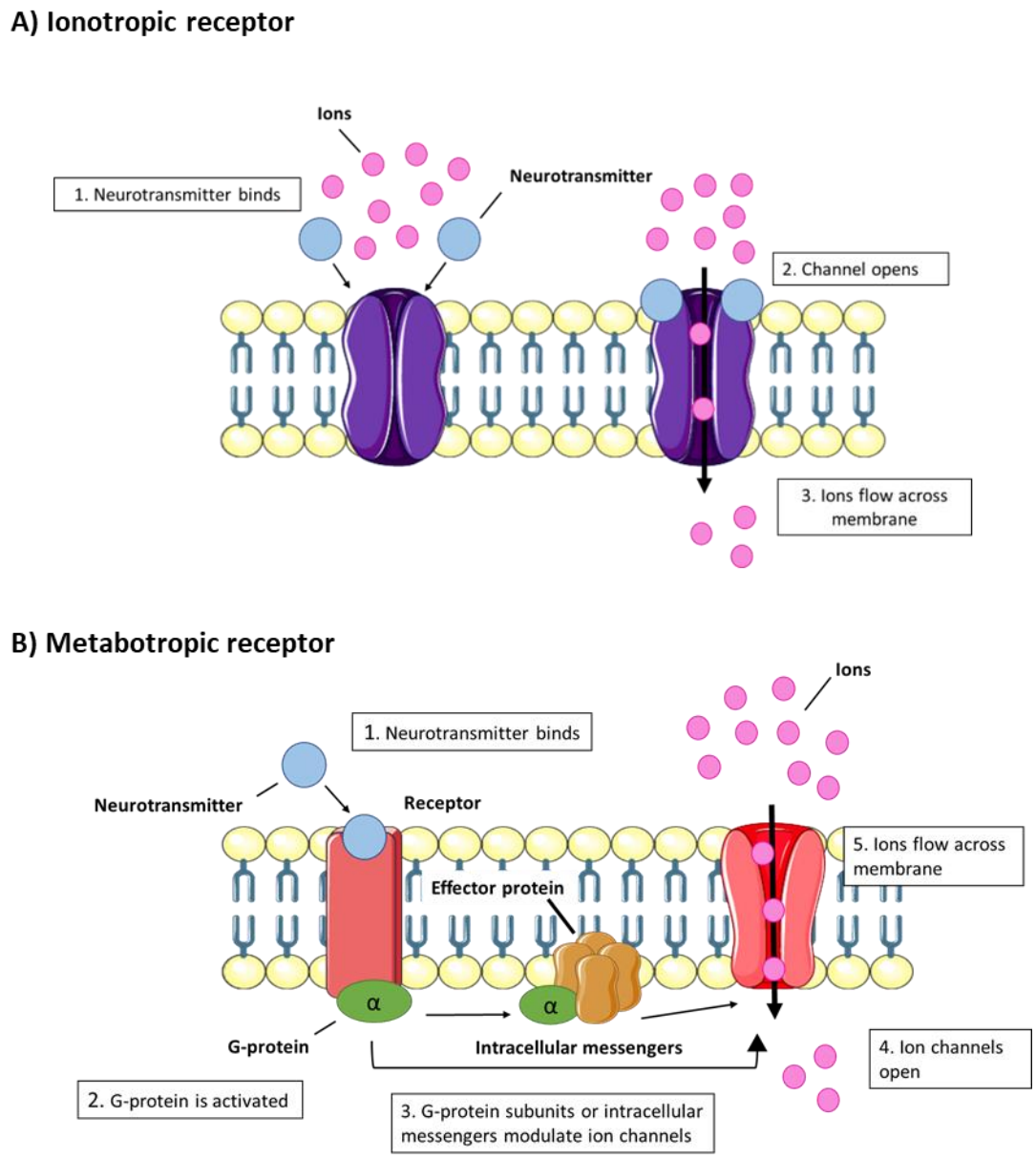

Figure 1.12. Schematic of ionotropic receptor (A) and metabotropic receptor (B). Ionotropic receptors and metabotropic receptors are considered to be the two types of neurotransmission receptors within the brain and peripheral nervous system (Roth, 2019). Ionotropic receptors are ligand-gated ion channels, where the binding of neurotransmitters leads to the activation of the ion channel (A) (Hinton & Johnston, 2018). Whereas, metabotropic receptors are G-protein coupled receptors that require secondary messengers to indirectly manage the activity of ions (B) (Roth, 2019).

GABA has three distinctive receptor sub-families; $GABA_A$, $GABA_B$ and $GABA_C$ (Li et al., 2016). Both $GABA_A$ and $GABA_C$ receptors are ionotropic receptors (Kannampalli & Sengupta, 2015) that are part of the superfamily of neurotransmitter-gated ion channels that include strychnine-sensitive glycine, nicotinic acetylcholine and $5HT_3$ receptors (Chebib & Johnston, 1999). Whereas, $GABA_B$ receptors are metabotropic (Kannampalli & Sengupta, 2015). When GABA binds to one of these receptors, the effects cause a decrease in excitability of the pre-synaptic or post-synaptic cell (Hinton & Johnston, 2018).

$GABA_A$ receptors are responsible for the most rapid inhibitory synaptic transmission within the vertebrate CNS (Goetz et al., 2007). $GABA_A$ receptors are permeable to Cl^- and HCO_3^-. The activation of $GABA_A$ receptors is neither inherently excitatory nor inhibitory, as the flow of ions through the pore depends on the membrane potential of the cell at the time, and the concentration gradient of Cl^- and HCO_3^- ions (Herbison & Moenter, 2011). The $GABA_A$ receptor is a major molecular target for drugs that work on the CNS. Some of these drugs include benzodiazepines, barbiturates, channel blockers, and potentially ethanol (Olsen & DeLorey, 1999).

Unlike $GABA_A$ receptors, $GABA_B$ receptors are always inhibitory. They were first recognised for their insensitivity to $GABA_A$ receptor antagonist bicuculline (Johnston, 1996). Activation of $GABA_B$ receptors mediates inhibitory effects via the activation of inwardly rectifying K^+ channels, which leads to the inhibition of voltage-gated Ca^+ channels (Wang & Lambert, 2000). The effects that follow $GABA_B$ activation include the inhibition of neurotransmitter release and the regulation of excitatory neurotransmission (Bettler et al., 2004). Although activation by $GABA_B$ agonists causes inhibition of adenylyl cyclase and reduction of intracellular cAMP levels (Calver et al., 2003), both the inhibition and enhancement of cAMP levels by $GABA_B$ receptors have been confirmed *in vivo* (Hashimoto & Kuriyama, 1997).

1.5.8. Bicuculline

Bicuculline is a convulsant alkaloid from *Dicentra cucullaria* (Hinton & Johnston, 2018). Bicuculline is known for its ability to reduce the effects of GABA, while not interfering with the action of glycine (Johnston, 2013a). As bicuculline is a potent competitive antagonist of $GABA_A$ receptors, it inhibits the binding of GABA to $GABA_A$ receptors. However, GABA also competitively inhibits bicuculline binding to these receptors (Andrews & Johnston, 1979). $GABA_A$ receptors can be defined by the antagonistic effects of bicuculline and their insensitivity to baclofen (Johnston, 1996), a selective $GABA_B$ receptor agonist (Beaurepaire, 2018).

Bicuculline has been used in the identification of potential drug compounds. For example, because it can induce seizures by inhibiting $GABA_A$ receptor function (Velíšek, 2017), it is a suitable model for screening anticonvulsant drugs, particularly drugs that act on $GABA_A$ receptors (Coppola & Moshé, 2012). A study by Frye and Breese also shows that bicuculline reduces ethanol-induced motor impairment (Frye & Breese, 1982). The half-life of bicuculline is 45 minutes at physiological pH (Jones et al., 2020).

1.5.9. Glycine

Although glycine is labelled as one of the major inhibitory neurotransmitters in the CNS (Ito, 2016), depending on its location in the CNS, it can function as both an inhibitory and excitatory neurotransmitter (de Bartolomeis et al., 2020). Glycine is the simplest of the amino acids (Vannier & Triller, 1997), and although is widespread throughout the mammalian CNS (de Bartolomeis et al., 2020), it is a non-essential amino acid (Vannier & Triller, 1997). The half-life of glycine is 26 minutes – 245 minutes (Hahn, 1993).

Much like $GABA_A$ receptors, glycine receptors (GlyRs) are also ionotropic ligand-gated chloride ion channels (Ito, 2016). However, there are two types of distinctive ligand-gated ion channels that glycine can activate: chloride-permeable inhibitory GlyRs and cation selective excitatory glutamate-gated channels (NMDARs) (de Bartolomeis et al., 2020). When GlyRs are activated, the postsynaptic membrane becomes hyperpolarised due to an influx in chloride ions, thereby reducing neuronal excitability (Dutertre et al., 2012). The inhibitory function of GlyRs is crucial in the control of numerous physiological processes, including, muscle tone, sensory processing, motor coordination and pain (Burgos et al., 2016).

1.5.10. Ethanol

Ethanol is the main ingredient in alcoholic beverages, therefore we used this as a substance of abuse, to establish whether ethanol has an effect on *L. variegatus* and whether *L. variegatus* can develop a tolerance to a substance of abuse. As ethanol is extremely water soluble and has a low molecular weight (Pereira et al., 2015), it readily passes through biological membranes (Wilson & Matschinsky, 2020) and the blood-brain barrier reaching the CNS (Pereira et al., 2015). In humans, ethanol is metabolised by alcohol dehydrogenase (ADH), which is an enzyme responsible for oxidising endogenous alcohol produced in the gut and exogenous ethanol (Cederbaum, 2012), however, similar mechanisms of metabolism have also been reported in vertebrate and invertebrate species (Wolf &

Heberlein, 2003). Ethanol influences several neurotransmitter systems in the CNS, such as GABA, dopamine, serotonin, opiates and glutamate (Diamond & Gordon, 1997), however, not all the effects executed by ethanol are understood (Dahchour & De Witte, 2000). Several studies, for example, have suggested that ethanol increases $GABA_A$ responses, whereas other studies have not detected any ethanol-induced increase in $GABA_A$ responses (Dahchour & De Witte, 2000). Although earlier ethanol studies were based on the theory that lipid membranes were the primary targets of ethanol, there is strong evidence indicating that the primary targets of ethanol are membrane proteins, particularly receptors (Spanagel, 2009). There are several receptors within the CNS that have presumed ethanol-binding sites, such as $GABA_A$, NMDA receptors, GlyRs, nicotinic acetylcholine receptors (nACh) and 5-HT_3 (Spanagel, 2009). The half-life of ethanol is 4-5 hours (Cleveland Clinic, 2021). However, the process of ethanol elimination is zero-order kinetics, meaning that a constant amount of ethanol is eliminated from the body per unit time (Jones, 2019). Overall, it takes around 25 hours for the body to eliminate ethanol completely (Cleveland Clinic 2021). Studies suggest a high-affinity ethanol binding site on GABAa receptors (α4, α6, β3, and δ subunits), meaning they are sensitive to concentrations of ethanol as low as 3 mM (Wallner et al., 2003).

1.5.11. 2,4-dinitrophenol

Similar to ethanol, 2,4-dinitrophenol (DNP) has no clinical or physiological use but instead is used socially as a weight-loss drug. DNP is a toxic benzene-based chemical (Colman, 2007) that is popular amongst the slimming and bodybuilding community (Thomas, 2018). DNP is often marketed as a 'safe weight loss agent' (Ost et al, 2017), as it induces fat-burning effects without the need for calorie control (Petróczi et al, 2015). However, the side effects of DNP include hyperthermia and death (Takahashi et al, 2009) and reports have shown that between 2019 and 2022 there were 15 cases of systemic DNP exposure. Since 2007 there have been 135 cases of systemic DNP exposure discussed, of which 25 patients have died (National Poisons Information Service, 2020).

DNP is a protonophore and produces its toxic effects by chemically uncoupling oxidative phosphorylation via the movement of protons through the mitochondrial lipid bilayer (Goldgof et al, 2014). This results in an increase in lipid metabolism and the release of energy as heat (Takahashi et al, 2009). DNP mimics the uncoupling effects of activated uncoupling proteins, resulting in non-specific uncoupling in all tissues (Ost et al, 2017).

Uncoupling protein 1 (UCP1) is important for maintaining both core body temperature and the control of energy intake and expenditure (Fedorenko et al, 2012). UCP1 inhibits the H+ proton gradient across

the inner mitochondrial membrane, resulting in the uncoupling of the electron transport train from adenosine triphosphate (ATP) synthesis (Ost et al, 2017). Energy is then released as heat when H+ re-enters the mitochondrial matrix, this is termed a proton leak (Figure 1.13) (Fedorenko et al, 2012).

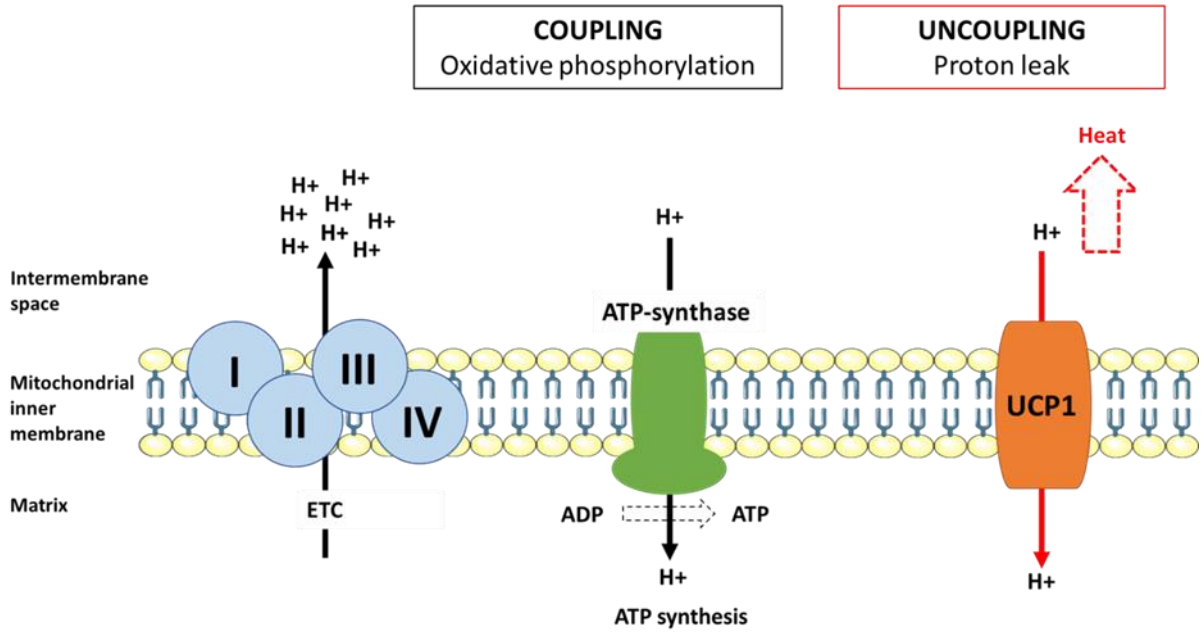

Figure 1.13. UCP1 function in the mitochondrial respiratory chain (MRC). During respiration a proton gradient is generated by the flow of protons pumped through the MRC complexes. The energy of the proton gradient fuels ATP synthesis via the ATP-synthase complex. UCP1 reduces this by the re-entry of H^+ into the matrix, resulting in energy being dissipated as heat (Adapted from: Brondani et al, 2012).

As DNP is able to inhibit ATP synthesis by the uncoupling of OXPHOS, the body tries to compensate for this via gluconeogenesis, glycolysis and lipolysis (Tewari et al, 2009). With a decrease in ATP production, Ca2+ transport is reduced, resulting in the accumulation of intracellular Ca2+, causing muscle contractions and heat production (Kopec et al, 2019). The increase in body temperature can result problematic symptoms such as seizures, coma, kidney failure, bone marrow failure and muscle damage (Thomas, 2018). These effects are complicated to treat and often the end result is death (Thomas, 2018). The half-life of DNP is 10.3 hours (Freeman et al., 2021).

Previous findings by Plater and Harrison at the University of Aberdeen proved haloperidol to be capable of adjusting the chemical structure of DNP. As DNP has a pKa of 4.0, it is considered a very acidic drug, being around 8-10 times more acidic than acetic acid (Plater & Harrison, 2019a). However, haloperidol has a pKa of 8.66 (El Tayar et al., 1985), meaning it is a basic drug. Drugs used in the treatment of the central nervous system are typically basic, this allows them to form an acid-base

complex with DNP (Figure 1.14) in the hope that it will reduce the availability of DNP in the system, thus easing the symptoms of toxicity (acid + base = salt) (Plater & Harrison, 2019).

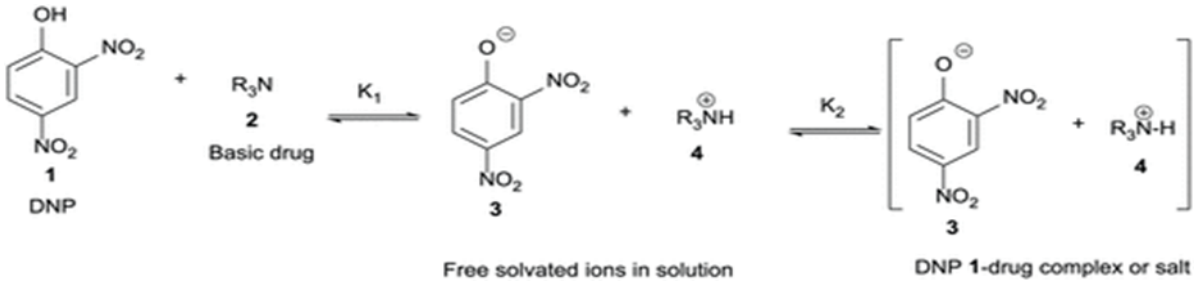

Figure 1.14. Schematic proposing the binding of (1) DNP with a (2) basic drug to give solvated ions in solution then a (3 & 4) acid-base complex or salt (Plater & Harrison, 2019).

We chose the drugs and compounds above as they are structurally diverse and have different pharmacodynamic properties. Some of compounds we chose for this project are endogenous in humans, for example dopamine, GABA, glycine, and ethanol in small concentrations (Sheffler et al., 2023; YuM, 1986). These were used as a proof of concept to determine whether compounds that effect human behaviour would have an effect on *L. variegatus*, which could pave the way for future research into whether *L. variegatus* possess similar receptor homologs to humans. We also chose drugs that are exogenously administered that are known to cause physiological effects on the human body with known pharmacokinetic and pharmacodynamic profiles. Again, these drugs such as lidocaine, quinine, dantrolene, haloperidol, sulpiride, bicuculline, were used as proof of concept to determine whether there would be any changes in *L. variegatus* behaviour and movement. 2,4-dinitrophenol was chosen as a substance of abuse. Our aim with DNP is to reverse any toxic effects we observe by administering it with a concurrent therapy.

1.6. Aims and objectives

Animal models in research are important as they contribute to our knowledge of human disease and drug discovery and development (Denayer et al., 2014). However, there is framework put in place to replace, reduce and refine the number of animal models used in research. Here we look to develop the aquatic oligochaete worm, *Lumbriculus variegatus*, as a novel *in vivo* model for pharmacology by observing the behavioural effects of different drugs and compounds.

Throughout this project, we aim to:

- Develop assays which will allow us to observe the effects of drugs on *L. variegatus* stereotypical behaviours and free locomotion.
- Determine the behavioural effects of drugs with specific mechanisms of action, such as channel blockers, neurotransmitters and drugs that antagonise the specific neurotransmitters chosen for this project.
- Determine the behavioural effects of substances of abuse, and whether *L. variegatus* are capable of developing tolerance.
- Utilising and determining whether *L. variegatus* could be used as a model for toxicological studies by exposing them to a toxic substance followed by a potential antidote drug to see if they will recover from any toxic effects.
- Further validating *L. variegatus* as an *in vivo* model for pharmacology by developing suitable assays for protein extraction and quantification, and DNA extraction and quantification.

2. Materials & Methods

2.1. Safety

All waste was disposed of according to manufacturer's instructions. All experimental procedures were analysed, and formal risk assessments were conducted and registered in control of substances hazardous to health (COSHH) forms.

2.2. Reagents and Solutions

Table 2.1. List of Reagents. List of reagents and manufacturers.

Reagent	**Supplier**	**Storage**
2,4-dinitrophenol (DNP)	Sigma-Aldrich	Room temp
Bicuculline	Sigma-Aldrich	-20°C
Bovine serum albumin (BSA)	Thermo Scientific	2-4°C
Bradford assay	Thermo Scientific	2-4°C
Bromophenol blue	Sigma-Aldrich	Room temp
Calcium nitrate tetrahydrate	Duchefa Biochemie	Room temp
Dantrolene	Sigma-Aldrich	Room temp
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Room temp
Dopamine	Sigma-Aldrich	2-4°C
DTT	Melford	2-4°C
Ethanol	Fisher Chemical	Room temp
GABA	Sigma-Aldrich	Room temp
Glycerol	Melford	Room temp
Glycine	Sigma-Aldrich	Room temp
Haloperidol	Sigma-Aldrich	Room temp
HEPES	Melford Laboratories	Room temp
Hydrochloric acid	Sigma-Aldrich	Room temp
Lidocaine	Sigma-Aldrich	Room temp
Magnesium sulphate heptahydrate	Duchefa Biochemie	Room temp
NP-40	Sigma-Aldrich	Room temp
Protease inhibitor cocktail (PIC)	Calbiochem® 539134	-20°C
Potassium chloride	Melford Laboratories	Room temp
Quinine	Sigma-Aldrich	Room temp
SDS	Sigma-Aldrich	Room temp
Sodium chloride	Melford Laboratories	Room temp
Sodium hydroxide	Sigma-Aldrich	Room temp
Sulpiride	Sigma-Aldrich	2-4°C
Tris Hydrochloride	Melford	Room temp

2.3. Storage and preparation of drugs and solutions

2,4-dinitrophenol

DNP was dissolved in 100% DMSO to give a 1 mM stock concentration. This was then diluted into working concentrations of 0.5 – 50 mM using 100% DMSO and added to artificial pond water at a final concentration of 0.5% DMSO.

Bicuculline

Bicuculline was dissolved in 100% DMSO to make a 100 mM stock and stored at -20^{o}C. Upon use, the stock was thawed and diluted with an additional 50 µL of DMSO to give a 50 mM stock concentration. This was then diluted into working concentrations of 2.5 - 250 µM using 100% DMSO and added to artificial pond water at a final concentration of 0.5% DMSO.

Coomassie Brilliant Blue

60-80mg of Coomassie Brilliant Blue G-250 was diluted in 1 L of ddH_2O. This was stirred for 2-3 hours at room temperature to dissolve before 3mL of concentrated hydrochloric acid was added to give a concentration of 35mM. This was stored away from light at room temperature.

Dantrolene

Dantrolene was dissolved in 100% DMSO to give a 10 mM stock concentration. This was then diluted into working concentrations of 0.5 – 50 µM using 100% DMSO and added to artificial pond water at a final concentration of 0.5% DMSO.

Dopamine

Dopamine was dissolved in artificial pond water to give a 100 mM stock concentration. This was then diluted into working concentrations of 10 – 100 mM using artificial pond water.

Ethanol

Ethanol was diluted into working concentrations of 25 – 500 mM using artificial pond water.

GABA

GABA was dissolved in artificial pond water to give a 100 mM stock concentration. This was then diluted into working concentrations of 0.1 – 100 mM using artificial pond water.

Glycine

Glycine was dissolved in artificial pond water to give a 500 mM stock concentration. This was then diluted into working concentrations of 50 – 500 mM using artificial pond water.

Haloperidol

Haloperidol was dissolved in 100% DMSO to give a 20 mM stock concentration. This was then diluted to working concentrations of 1 – 100 µM using 100% DMSO and added to artificial pond water at a final concentration of 0.5% DMSO.

Lidocaine

Lidocaine was dissolved in artificial pond water to give a 1 mM stock concentration. This was then diluted into working concentrations of 0.001 – 1 mM using artificial pond water.

Quinine

Quinine was dissolved in 100% DMSO to give a 200 mM stock concentration. This was then diluted into working concentrations of 0.001 – 1 mM using 100% DMSO and added to artificial pond water at a final concentration of 0.5% DMSO.

Sample buffer

6x sample buffer was made using Tris Hydrochloride, hydrochloric acid was added until the solution was pH 6.8. 2x sample buffer was made by diluting 6x sample buffer 1:3 times with sterile ddH2O.

Radio immunoprecipitation assay (RIPA)

To make up 10mL of RIPA lysis buffer 29.22g of NaCl was dissolved in up to 100mL of ddH_2O to make a 5M NaCl solution. 10 mL of NP-40 was then added to 100mL of ddH_2O to make a 10% solution, and a 10% sodium deoxycholate solution was made by weighing out 10g of sodium deoxycholate and adding up to 100mL of ddH_2O. A 1M Tris pH 8.0 solution was then made by dissolving 12.114g of Tris base in up to 100mL of ddH_2O, this was then made to a pH of 8.0 by adding hydrochloric acid. A 1M sodium hydroxide solution was then made by weighing out 2g of sodium hydroxide, this was then slowly added and dissolved in 50mL of ddH_2O. 10mL of RIPA was then made by combining 0.3mL of 5M NaCl solution, 1mL of the

10% NP-40 solution, 0.5mL of the 10% sodium deoxycholate solution, 0.1mL of 10% SDS and 0.5mL of 1M Tris pH 8.0, this was then made up to 10mL with ddH_2O.

The prepared RIPA lysis buffer was then aliquoted into 500 µL and stored at -20^oC. Before using RIPA, the protease inhibitor cocktail (PIC) (Calbiochem®) was added at a dilution of 1:1000 (0.5 µL).

Sulpiride

Sulpiride was dissolved in 100% DMSO to give a 200 mM stock concentration. This was then diluted into working concentrations of 25 – 200 mM using 100% DMSO and added to artificial pond water at a final concentration of 0.5% DMSO.

20 x TBS-T

A 1L 5M NaCl solution was made up by weighing out 292.2g of NaCl and adding up to 1L of ddH2O. 200mL 1M Tris buffer pH 8.0 was made using 24.2g of Tris base and adding up to ~175mL of ddH2O. This was then made to a pH of 8.0 by adding HCl acid. For the 20 x TBS-T solution 600mL of 5M NaCl solution, 200mL of Tris buffer pH 8.0 and 10g of Tween-20 were combined and ddH_2O was added to make a total volume of 1L. 20 x TBS-T was then diluted for 1x TBS-T before use.

2.4. *Lumbriculus variegatus* Culture

L. variegatus cultures were derived from ALFA Fish Foods and laboratory-reared in aquariums containing artificial pond water, as previously described by O'Gara *et.al* (2004). Artificial pond water was made using the following composition: 1 mM NaCl; 13 µM KCl, 4 µM $Ca(NO_3)\cdot 4H_2O$; 17 µM $Mg(SO_4)\cdot 7\ H_2O$; 71 µM HEPES buffer. The aquaria were kept at room temperature and *L. variegatus* were exposed to a 16:8-hour light-dark cycle. The artificial pond water in the aquariums was filtered and aerated using air stones. Cultures were fed weekly with TeraMin flakes and 10 mg/L spirulina. Cultures were maintained for at least 3 months before experimentation and individual worms used in experiments were selected at random and lacked any obvious morphological anomalies. *L. variegatus* were removed from aquariums and placed into 6-well plates containing artificial pond water 8-24 hours before any experimentation.

2.5. Stereotypical Movement Assay

18-24 hours before experimentation, individual worms were placed one per well into 6-well plates containing artificial pond water. Immediately before experimentation, the artificial pond water in the wells was replaced and the worm's ability to perform stereotypical behaviours pre-drug exposure was tested and recorded (Baseline). *L. variegatus'* ability to perform body reversal and helical swimming was assessed by stimulating the anterior and posterior ends, respectively. Stimulation of the posterior and anterior end was carried out using a clean 20-200 µL pipette tip, with a 5-10 second interval between stimuli. Movement was recorded using a scoring sheet (see Appendix 1) and movement was scored as 1 = No movement, 2 = Some movement and 3 = Full stereotypical movement. The artificial pond water was then removed, and a vehicle (artificial pond water only or 0.5% DMSO in artificial pond water) or drug solution was added. Stimulation was carried out again after 10 minutes exposure to vehicle/drug solution (Drug exposure). The vehicle or drug solution was then removed and wells were rinsed to remove any residual drug compounds with artificial pond water. This was then removed and fresh artificial pond water added back into the wells and *L. variegatus's* ability to perform stereotypical behaviours was re-tested 10 minutes (Rescue 10 mins) and 24 hours (Rescue 24 hrs) after drug or vehicle removal.

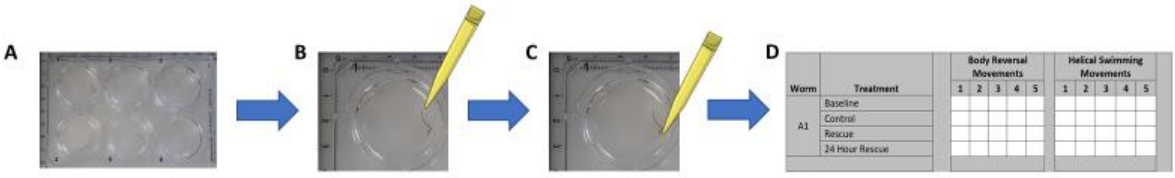

Figure 2.1. Measuring stereotypical movement of *Lumbriculus variegatus*. (A) *L. variegatus* are plated in 6-well plates 18 – 24 h before the experiment begins. *L. variegatus.* The ability of *L. variegatus* to perform stereotypical behaviours was then recorded by alternate stimulation with 20 to 200 µL pipette tip of the **(B)** anterior or **(C)** posterior end of *L. variegatus* for a total of 5 times per end, with a 5 – 10 second interval between stimuli. **(D)** The ability to perform stereotypical movement was scored as 1 = No movement, 2 = Some movement, 3 = Full Stereotypical Movement., as previously described by Drewes, 1999 (Drewes, 1999). A-D is repeated for each *L. variegatus* before exposure to drug compounds to give the baseline ability to perform these movements. *L. variegatus* are then tested again 10-minutes after incubation with drugs and 10-minutes and 24-hours in artificial pond water only. Data are expressed as a ratio of the movement score after exposure relative to the baseline movement score (adapted from Seeley et.al, 2021).

2.6. Free Locomotion Assay

18-24 hours prior to experimentation, individual worms were placed one per well into 6-well plates containing artificial pond water. Immediately before experimentation, the artificial pond water in the wells was replaced with 2 mL of fresh artificial pond water to limit movement in the z-axis. Baseline free locomotion was then recorded using rapid, sequential image collection using a 13-megapixel camera at the rate of 1 image per second for 50 seconds. The artificial pond water was then removed and replaced with drug treatment or vehicle for 10 minutes before collecting more images. The drug solution was removed, each well was rinsed as in the stereotypical movement assay, and replaced with fresh, untreated artificial pond water. *L. variegatus* free locomotion was then recorded using image collection again 10 minutes and 24 hours after drug or vehicle removal.

Images collected during this assay were analysed using ImageJ. The 50 images collected were compressed together to create a z-stack image and the area of known distance within each z-stack was measured (Figure 2.2). This allowed us to measure the total area covered by *L. variegatus* at Baseline, Drug exposure, Rescue (10 min) and Rescue (24 hrs).

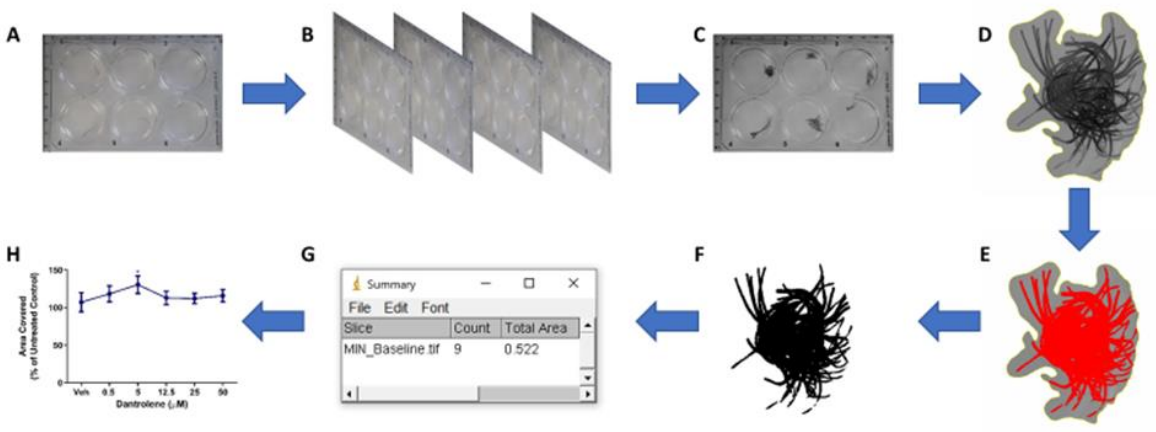

Figure 2.2. Measuring free locomotion of *Lumbriculus variegatus*. (A) *L. variegatus* are plated in 6-well plates 18 – 24 h before the experiment begins **(B)** 50 images are then collected at 1-second intervals **(C)** Images are then arranged into a *z*-stack and the scale is set to an area of known distance within the z-stack. **(D)** Each *L. variegatus* is isolated using freehand selection and **(E)** thresholds are adjusted to only select *L. variegatus* before **(F)** background is then removed. **(G)** The total area covered by each *L. variegatu s*can then be calculated using the set scale and **(H)** graphed for presentation and analysis. A-F is repeated for each *L. variegatus* to give the baseline movement before exposure to drug compounds, 10 minutes after incubation with drugs and 10 mins and 24 h in artificial pond water only. Data are expressed as a percentage of baseline controls. (Taken from Seeley et.al, 2021).

2.6.1. Acute Functional Tolerance

Acute functional tolerance was determined by measuring the free locomotion of *L. variegatus* at 20-minute intervals for 210 minutes during continuous exposure to 500mM ethanol.

2.7. Protein extraction

Protein was extracted from whole worms, 1 worm per protein sample. *L. variegatus* were separated into 6-well plates 18-24 hours before experiments. All solutions and samples were kept on ice throughout. Radio immunoprecipitation assay (RIPA) was thawed on ice and a 1:1,000 dilution of protease inhibitor cocktail (Calbiochem®) was added. Worms were transferred to 1.5mL Centrifuge tubes, one worm per Centrifuge tube, and all pond water was removed. The worms were then rinsed twice with 200 µL of ice-cold artificial pond water and artificial pond water removed. Following this, 20 µL of fresh ice-cold artificial pond water was then pipetted into each Centrifuge tube containing the worms. Each worm was homogenised for 10-20 seconds using a tissue homogeniser (Argos Technologies). These samples were then snap frozen at -80^{o}C for 60 minutes.

80 µL of RIPA + PIC was then added to each sample and left to lyse on ice for 30 minutes. Samples were spun at maximum speed for 15 minutes in a 4^{o}C centrifuge. The supernatant was then transferred to sterile Centrifuge tubes and the pellet discarded.

2.8. Protein Quantification

5 µL of extracted protein was used for protein quantification. To quantify protein according to Bradford (Bradford, 1976), a standard curve was made using bovine serum albumin (BSA) and Bradford assay. The 2mg/mL BSA was diluted to 0, 1, 2, 4, 8 and 10µg/mL. The absorbance on a spectrophotometer was set to 590nm and each BSA sample was measured. For *L. variegatus* protein quantification each cuvette contained 1 mL of Bradford and 1 µL of supernatant. The spectrophotometer was blanked with a cuvette containing 1 µL RIPA + PIC and 1 mL of Bradford. The protein absorbance of each sample was measured, and the data was entered into excel. The concentration of each sample was then calculated using the following equation:

$$\boldsymbol{y = mx + c}$$

30 µg of protein was combined with RIPA and 2x sample buffer (117mM Tris pH 6.8, 10% glycerol, 3.3% SDS, 200mM DTT and 0.04% bromophenol blue). This was heated to 95^{o}C for 5 minutes. Samples were then stored at -20^{o}C until further usage.

2.9. SDS PAGE, protein transfer and staining

Proteins were separated by performing sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Invitrogen Mini Gel Tank (Invitrogen) using Novex® Tris-glycine SDS running buffer (Invitrogen) as per manufacturer's instructions. Samples containing 30 µg of protein were loaded onto polyacrylamide gels and subjected to SDS-PAGE at a constant voltage of 100 V. Page Ruler Plus Protein Ladder (Thermo Scientific) was used as a reference for protein size (Figure 2.3).

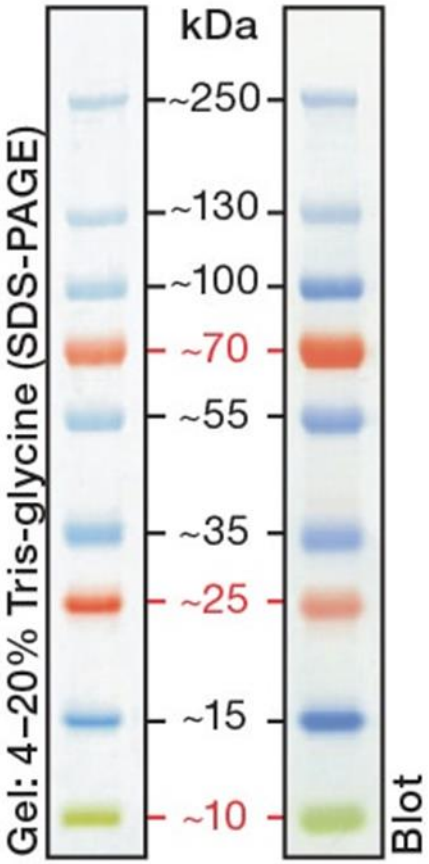

Figure 2.3. PageRuler Plus Stained Protein Ladder from ThermoFisher. Nine bands of colour-stained protein ranging in weight from 10-250 kDa. This protein ladder was used as a reference for protein size in SDS-PAGE.

Proteins were transferred from the gel onto a membrane using Invitrogen Mini Blot Module as per the manufacturer's instructions.

250mL of 1x transfer buffer was prepared. Two pieces of filter paper and two sponges were soaked in 1x transfer buffer. The membrane was carefully cut to size and placed in methanol to activate it. The cathode core (-) was placed on a flat surface and a module sandwich was assembled (Figure 2.4). The protein was transferred for 60 minutes at a constant voltage of 10V.

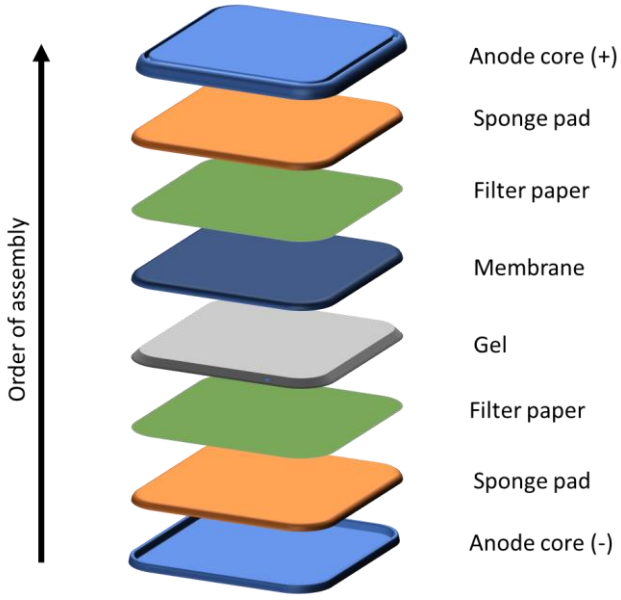

Figure 2.4. Order of assembly of Mini Blot Module sandwich. The blot module sandwich was assembled as above, using Blotting Tweezers to handle the membrane and Blotting Roller to remove any air bubbles from layers of the sandwich, particularly between the membrane and gel.

2.9.1. Coomassie blue staining

The gel containing the protein ladder and protein samples was placed in a shallow microwave-friendly container and covered with Coomassie blue. This was heated for 10 seconds in the microwave before the container was transferred to a rocking shaker for 10 minutes. The gel was then rinsed with ddH2O and the Coomassie staining was repeated two more times in order for the protein bands to be as clear as possible.

2.9.2. Ponceau staining

Once the protein had been transferred to the membrane the membrane was placed in ponceau stain and left on a rocking shaker for 10 minutes. The ponceau stain was then removed and the membrane was rinsed in 1 x TBS-T solution 1-2 times until protein bands became clear.

2.10. DNA extraction

L. variegatus were separated into 6-well plates 18-24 hours before experimentation. All solutions and samples were kept on ice throughout this assay, and heating blocks and water baths were set before starting DNA extraction. DNA was extracted from whole worms using the E.N.Z.A.® Tissue DNA Kit (Omega Bio-Tek) per the manufacturer's instructions, with one exception: elution buffer was heated to 55^{o}C rather than 70^{o}C.

Worms were transferred to 1.5mL centrifuge tubes, one worm per centrifuge tube, and all pond water was removed. The worms were then rinsed twice with 200 µL of ice-cold artificial pond water. Following this, 20 µL of fresh ice-cold artificial pond water was then pipetted into each Centrifuge tube containing the worms. Each worm was homogenised for 10-20 seconds. 200 µL of TL buffer was then added to each sample, followed by 25 µL of proteinase K solution. The samples were then incubated at 55^{o}C for 30 minutes in a pre-heated water bath. The samples were then placed in a 4oC centrifuge where they were spun at maximum speed for 5 minutes. The supernatant was then carefully transferred to sterile 1.5mL Centrifuge tubes and pellets were discarded. 220 µL of BL buffer was added to each sample and the samples were incubated at 70^{o}C for 10 minutes. Following incubation, 220 µL of 100% ethanol was added to each sample, these were then transferred to 2mL collection tubes containing a HiBind® DNA Mini Column. Samples were then centrifuged at maximum speed for 1 minute, any filtrate was discarded. 500 µL HBC buffer diluted with 100% isopropanol was then added to each sample and these were centrifuged for 30 seconds at maximum speed. Both filtrate and collection tubes were then discarded, and HiBind® DNA Mini Column was inserted into a sterile 2mL collection tube. 700 µL of DNA wash buffer was then added, and the samples were centrifuged at maximum speed for 30 seconds, this was done twice discarding any filtrate between spins. The empty HiBind® DNA Mini Column was then centrifuged at maximum speed for 2 minutes to dry the column, and the HiBind® DNA Mini Column was then transferred into a nuclease-free 1.5mL microcentrifuge tube. Elution buffer was heated to 55^{o}C, and 100 µL of this was added to each sample, these were then left at room temperature for 2 minutes, before centrifuging at maximum speed for 1 minute. This was then repeated for a second elution. All eluted DNA samples were then stored at -20^{o}C.

2.11. DNA quantification

L. variegatus DNA samples were quantified using a Nanodrop spectrophotometer. 1 µL of each sample was pipetted onto the Nanodrop spectrophotometer pedestal and the arm was placed down. A reading was taken which determined both the 260:280 ratio and the 260:230 ratio of the DNA samples. The samples were tested and measured in triplicate, and the average was calculated and recorded.

2.12. *L. variegatus* disposal

L. variegatus were aspirated following assay end-points and euthanised by exposure to 70% ethanol and then incubated in virkon for 24 hours before disposal.

2.13. Statistical Analysis

Statistical significance was determined using GraphPad Prism 9. Data was analysed using either unpaired two-tailed t-tests for drug exposure conditions for both stereotypical movement and free locomotion or two-way ANOVA with Dunnett's post-test for 10-minute and 24-hour rescue time points. Drug exposure and rescue time points were compared to Baseline for each worm per condition. Statistical differences in acute functional tolerance were measured by one-way ANOVA with Dunnett's post-test compared to the 10 minutes of ethanol exposure. The threshold for statistical significance is $p<0.05$. Graph error bars show the mean ± standard error of the mean (SEM).

3. Results chapter 1: Investigating the effects of ion channel blockers in *L. variegatus*

Ion channels allow specific inorganic compounds to diffuse rapidly through the lipid bilayer of cells. As the ability to control ion fluxes through these channels is fundamental for many cell functions (Alberts et al., 2002), we sought to determine the effect of three distinct channel blockers on *L. variegatus* stereotypical and locomotor behaviours.

We firstly exposed *L. variegatus* to lidocaine, a voltage-gated sodium channel blocker and local anaesthetic. Voltage-gated sodium channels are primarily found in excitable cells, such as nerve, muscle and neuroendocrine cell types, and are responsible for action potential generation and propagation (Catterall et al., 2019).

We determined that lidocaine is able to significantly inhibited *L. variegatus* body reversal and helical swimming, Figure 3 A and B respectively, at 0.5 mM (p=0.0313) and 1 mM (p=0.0313). Effects shown at concentrations ≤0.5 mM were reversed after a 10-minute rescue period in drug-free artificial pond water, with no significant difference compared to baseline (p>0.05, Figure 3.1. C-D). The effect of 1 mM of lidocaine persisted 10-minutes after removal, still inhibiting body reversal (p=0.0115, Figure 3.1. C) and helical swimming (p=0.0035, Figure 3.1. D). After a 24-hour rescue period in drug-free artificial pond water, both movements returned to baseline level (p>0.05, Figure 3.1. C-D).

These dose-dependent responses were also observed in the free locomotion assay (Figure 3.1. E-G), as lidocaine reduced *L. variegatus* free locomotion by 76% at 0.5 mM (p<0.0001, Figure 3.1 F) and 86% at 1 mM. Much like the stereotypical movement assay, free locomotion returned to baselines levels 10-minute and 24-hours after drug exposure (p>0.05, Figure 3.1 G).

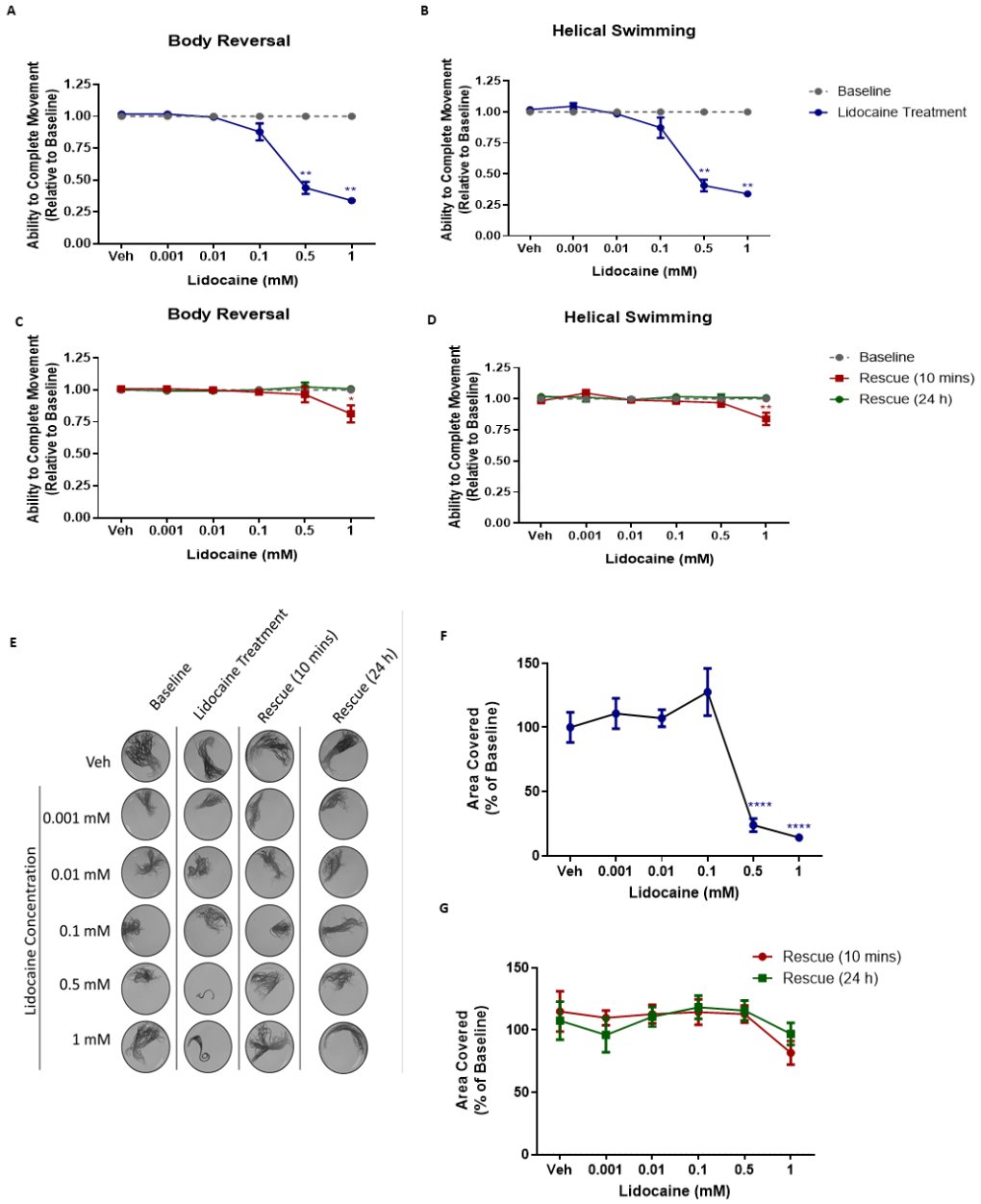

Figure 3.1. The effect of lidocaine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of Lidocaine (0.001 – 1 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Lidocaine was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of lidocaine on free locomotion was measured before lidocaine exposure (Baseline), after 10 minutes of exposure to 0.001 – 1 mM lidocaine (Lidocaine Treatment), 10 minutes after lidocaine removal (Rescue (10 mins)) and 24 hours after lidocaine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** lidocaine treatment and **(G)** removal of lidocaine for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: artificial pond water. **p*<0.05, ***p*<0.01, *****p*<0.0001.

Following on from lidocaine, we sought to determine the effect of a different ion channel blocker on *L. variegatus*. Quinine is a non-specific sodium and potassium channel blocker (Gisselmann et al., 2018). Potassium channels can be found in nearly all species, and play key roles in both excitable and non-excitable cells (Kuang et al., 2015).

As shown in Figure 3.2 A-B, quinine inhibited both body reversal and helical swimming at equimolar concentrations to lidocaine (0.5 mM and 1 mM). These effects persisted after 10-minutes and 24-hours in drug-free artificial pond water (p<0.0001, Figure 3.2 C-D).

When observing the free locomotion, the results were similar to the stereotypical movement assay. However, there was a 45% increase in movement at 0.01 mM (p=0.0006) which returned to baseline levels after 10-minutes in drug-free artificial pond water. Movement was decreased by 52% at 0.5 mM (p<0.0001, Figure 3.2 F) and by 90% at 1 mM (p<0.0001, Figure 3.2 F). As shown in Figure 3.2 G, *L. variegatus* free locomotion did not return to baseline levels after 10-minutes in drug-free artificial pond water as the movement was still decreased by 33% at 0.5 mM (p=0.0112) and by 84% at 1mM (p<0.0001). These effects persisted after 24-hours in drug-free artificial pond water with movement still decreased by 42% at 0.5 mM (p=0.0021) and 67% at 1 mM (p<0.0001).

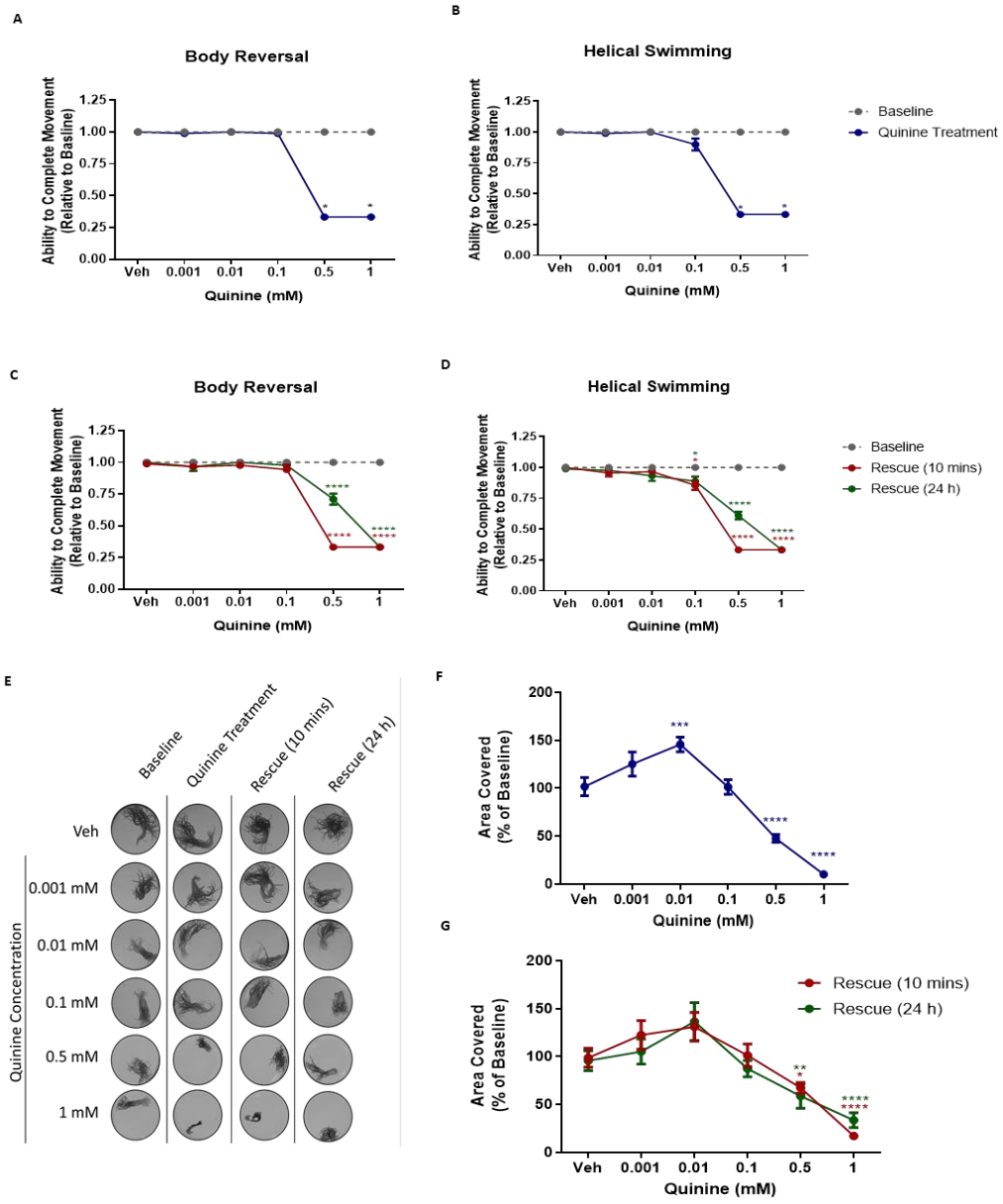

Figure 3.2. The effect of quinine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of quinine (0.001 – 1 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Quinine was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of quinine on free locomotion was measured before quinine exposure (Baseline), after 10 minutes of exposure to 0.001 – 1 mM quinine (Quinine Treatment), 10 minutes after quinine removal (Rescue (10 mins)) and 24 hours after quinine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** quinine treatment and **(G)** removal of quinine for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: 0.5% DMSO in artificial pond water. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Having observed *L. variegatus* behaviours when exposed to a voltage-gated sodium channel blocker and non-selective sodium and potassium blocker, we were interested to see whether a compound that alters calcium would affect *L. variegatus* behaviour and movement. To do so, we exposed *L. variegatus* to the ryanodine receptor antagonist, dantrolene (Krause et al., 2004). Ryanodine receptors are located in the sarcoplasmic reticulum membrane in cardiac and skeletal muscle and are crucial for the release of calcium from intracellular stores during excitation-contraction coupling (Lanner et al., 2010).

Dantrolene had no significant effect on *L. variegatus* stereotypical movements at ≤25 µM. However, body reversal was inhibited at 50 µM (p=0.0313, Figure 3.3 A-B) and these results persisted after 10-minutes in drug-free artificial pond water, as body reversal was still inhibited at 50 µM (p=0.0121, Figure 3.3 C). Figure 3.3 D shows after 10-minutes in drug-free artificial pond water, helical swimming was significantly reduced at concentrations of 25 µM (p=0.0088, Figure 3.3 D) and 50 µM (p=0.0081) compared to baseline levels. After 24-hours in drug-free artificial pond water, the effect of dantrolene on helical swimming persisted at concentrations of 25 µM (p=0.0290, Figure 3.3 D) and 50 µM (p=0.0015, Figure 3.3 D).

As shown in Figure 3.3 E-G, dantrolene did not have a significant effect on *L. variegatus* free locomotion, with the exception of 5 µM (p= 0.0341, Figure 3.3 F) where movement was increased by 30%. After 10-minutes and 24-hours in drug-free artificial pond water, this increase returned to baseline level ($p>0.05$, $n=8$).

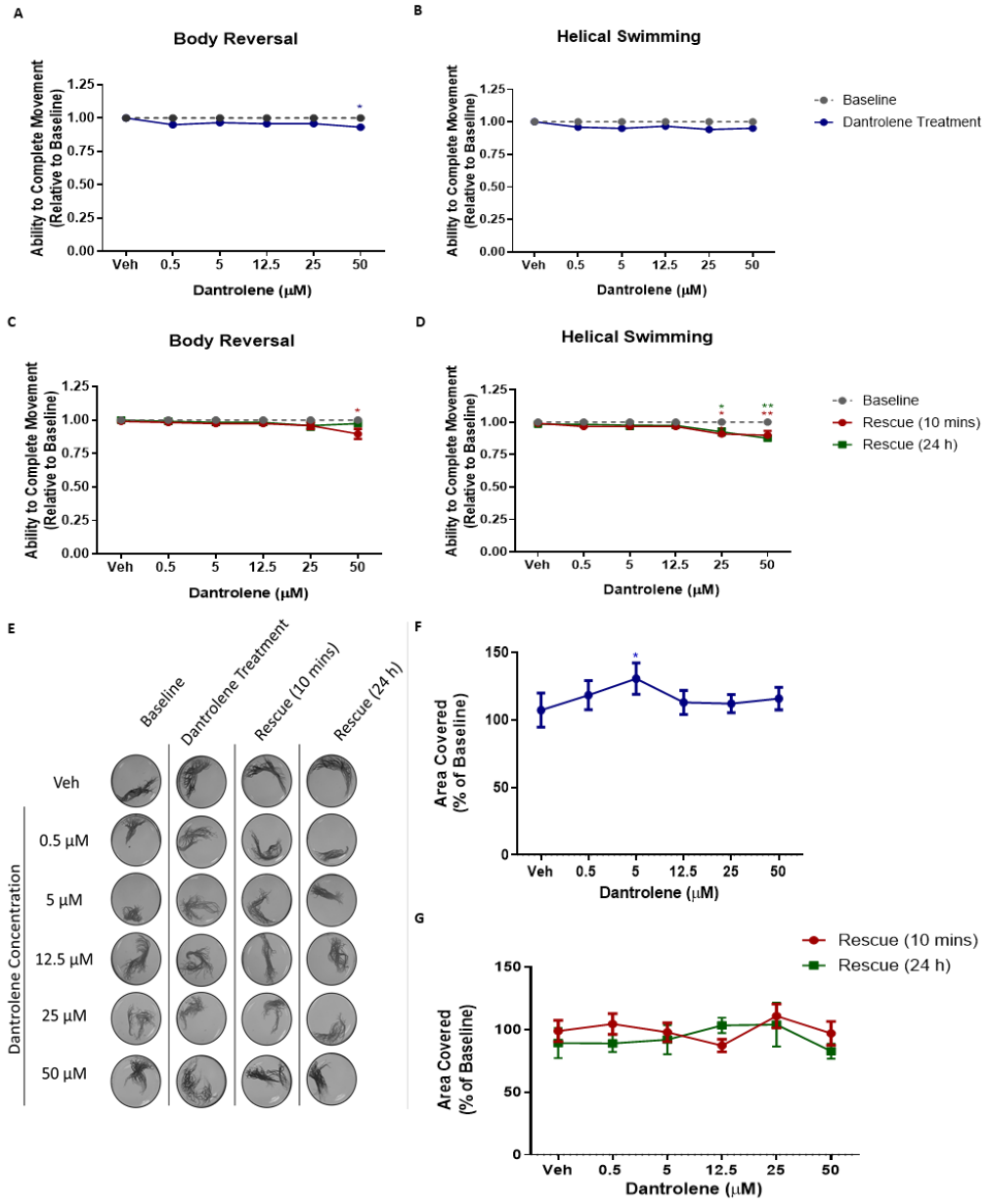

Figure 3.3. The effect of dantrolene on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of dantrolene (0 – 50 µM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Dantrolene was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of dantrolene on free locomotion was measured before dantrolene exposure (Baseline), after 10 minutes of exposure to 0 – 50 µM dantrolene (Dantrolene Treatment), 10 minutes after dantrolene removal (Rescue (10 mins)) and 24 hours after dantrolene removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** dantrolene treatment and **(G)** removal of dantrolene for 10 minutes and 24 hours are the mean, n=8 for each concentration. Experimental repeats were conducted in collaboration with Yusuf Hussein. Veh: 0.5% DMSO in artificial pond water. **p*<0.05, ***p*<0.01.

3.1. Chapter Summary

In this section, we show that several types of ion channel blockers affect the stereotypical movement and free locomotion of *L. variegatus*.

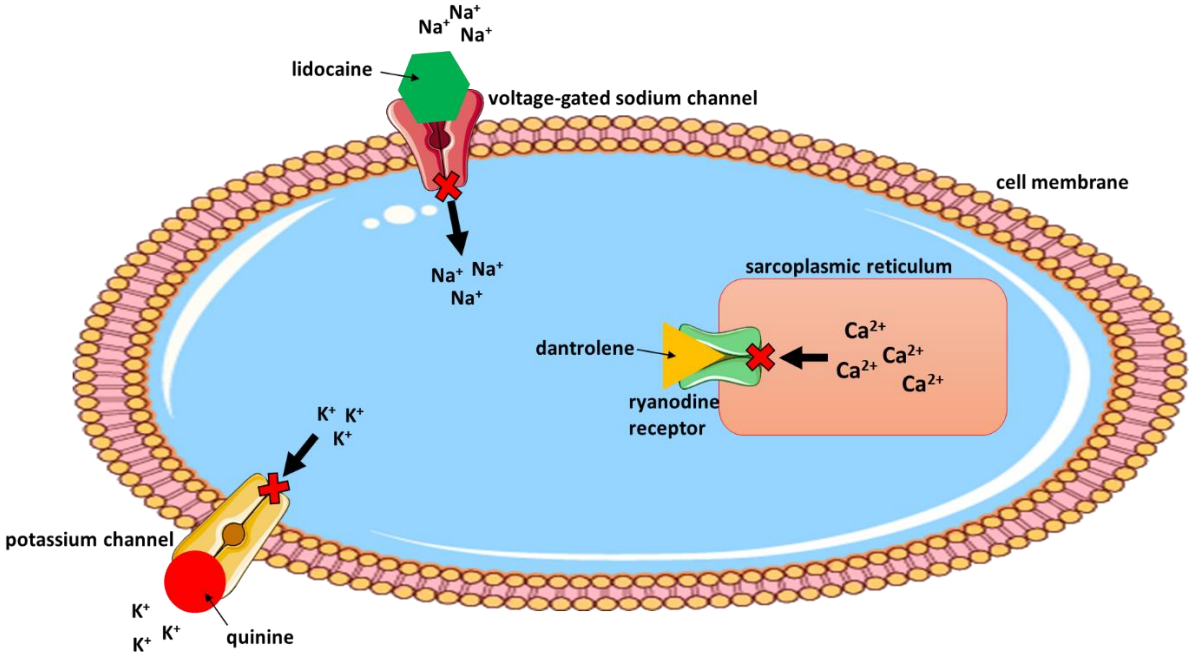

Figure 3.4. Schematic of channel blockers binding to different channels on cell membrane and inside the cell. Lidocaine (green), binds to voltage-gated sodium channel preventing the flow of Na^+ ions inward through the channel pore. Quinine (red), binds to potassium channel preventing the flow of K^+ ions outward through the channel pore. Dantrolene (yellow), binds to ryanodine receptors, inhibiting release of calcium from the sarcoplasmic reticulum and therefore decreasing intracellular calcium concentration (Image adapted from Benson, 2016; Sutherland, 2016).

4. Results chapter 2: Investigating exogenous inhibitory and excitatory neurotransmitters in *L. variegatus*

Ion channels open and close in response to stimuli (Wedegaertner, 2009). The main types of stimuli known to open ion channels are change in the voltage across the membrane, mechanical stress or the binding of a ligand (Alberts et al., 2002). The ligand can be a neurotransmitter, an intracellular mediator, such as an ion, or a nucleotide (Alberts et al., 2002). As such we wanted to see *if L. variegatus* would respond to the channels being activated, rather than inhibited. We did this by exogenous administration of neurotransmitters. We firstly exposed *L. variegatus* to the neuromodulator, dopamine (Guy-Evans, 2021).

As shown in Figure 4.1 A-B, dopamine significantly inhibited body reversal and helical swimming at ≥20 mM (p<0.01). These results persisted after 10-minutes in drug-free artificial pond water (Figure 4.1 C-D), with the addition of a significant change seen in body reversal at 10 mM (p=0.0002). These results returned to baseline levels after 24-hours in drug-free artificial pond water (p>0.05).

Similar to the stereotypical movement assay, we found that dopamine inhibited *L. variegatus* free locomotion at ≥20 µM (Figure 4.1 F). 20 mM was the lowest concentration at which a significant decrease in movement was observed, as movement was decreased by 47% (Figure 4.1 F). At the highest concentration of 100 mM movement was decreased by 40% (Figure 4.1 F). However, after 10-minutes in drug-free artificial pond water, this effect was also seen at 10 mM as movement decreased by 33% (p<0.0018, Figure 4.1 G). After 24-hours in drug-free artificial pond water, all concentrations returned to baseline levels (p>0.05), with the exception of 100 mM where movement was still decreased by 32% compared to baseline levels (p<0.0014, Figure 4.1 G).

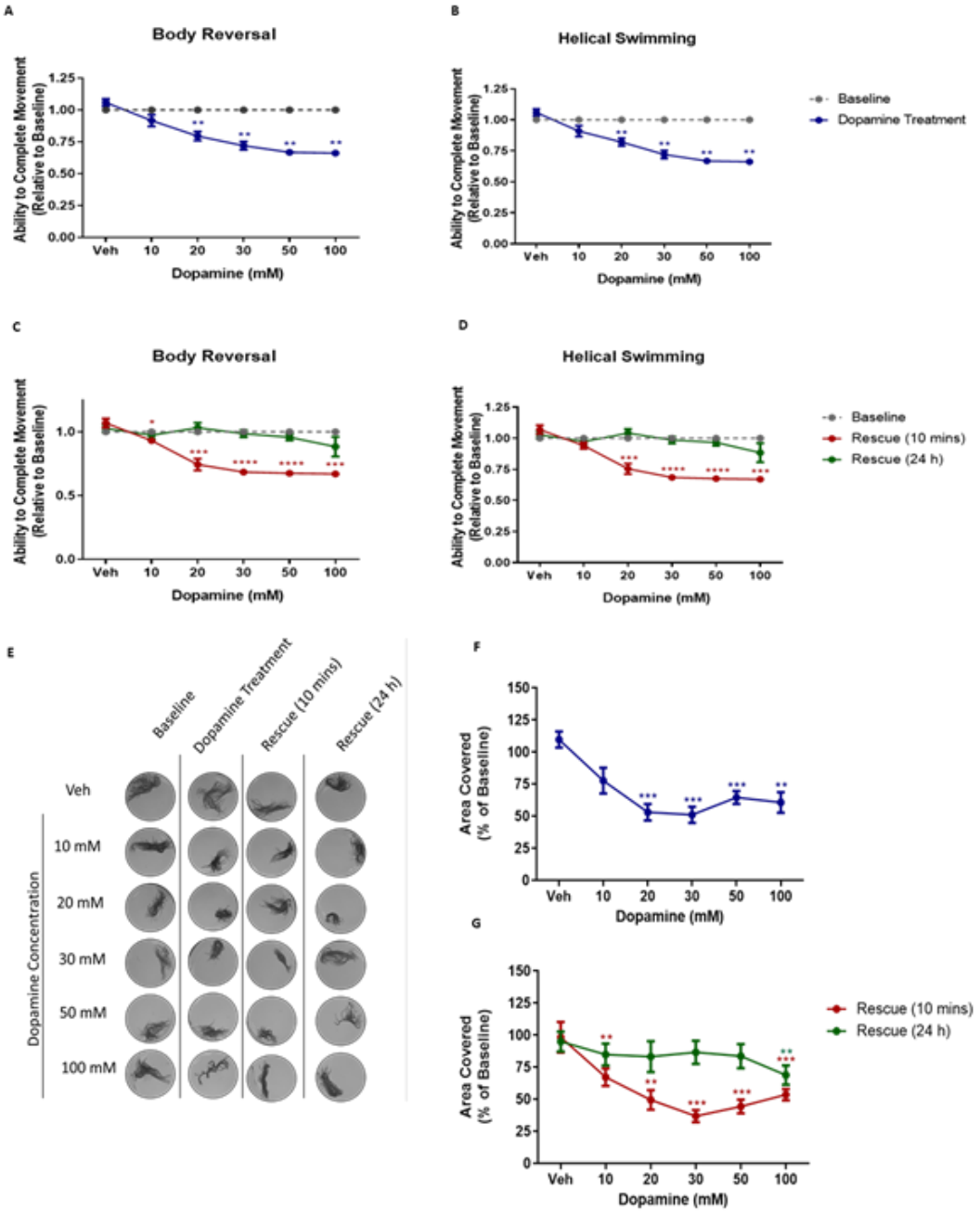

Figure 4.1. Effect of dopamine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of dopamine (10 – 100 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Dopamine was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of dopamine on free locomotion was measured before dopamine exposure (Baseline), after 10 minutes of exposure to 10 – 100 mM (dopamine Treatment), 10 minutes after dopamine removal (Rescue (10 mins)) and 24 hours after dopamine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** dopamine treatment and **(G)** removal of dopamine for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: artificial pond water. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

As dopamine had a significant effect on both *L. variegatus* stereotypical behaviour and free locomotion, we wanted to determine what, if any, dopamine receptors *L. variegatus* possess. We started by exposing *L. variegatus* to nonselective dopamine D_1/D_2 receptor antagonist, haloperidol (Kumari et al., 1999), to see whether it would have a similar, or opposite effect on *L. variegatus* behaviour as when we administered dopamine.

Figure 4.2 A-B show that haloperidol has a significant effect on *L. variegatus* body reversal and helical swimming at concentrations of 50 µM (p=0.0078) and 100 µM (p=0.0078). After 10-minutes in drug-free artificial pond water a decrease in body reversal compared to baseline was seen at 100 µM (p=0.0049, Figure 4.2 C), however, helical swimming was decreased at 50 µM (p=0.0421, Figure 4.2 D) and 100 µM (p=0.0015, Figure 4.2 D). After 24-hours in drug-free artificial pond water, all results returned to baseline levels, with the exception of helical 100 µM (p=0.0461, Figure 4.2 D).

Unlike the stereotypical behaviour, haloperidol increased *L. variegatus* free locomotion by 9% at 10 µM (Figure 4.2 F). Figure 4.2 F shows that there was a decrease in *L. variegatus* free locomotion at concentrations ≥25 µM. Movement was decreased by 19% at 25µM (p=0.0295), 17% at 50 µM (p=0.0389), and 51% at 100 µM (p=0.0007). These results persisted after 10-minutes in drug-free artificial pond water (Figure 4.2 G). After 24-hours in drug-free artificial pond water results returned to baseline levels, with exception of 25 µM and 50 µM (Figure 4.2 G). Movement was decreased by 18% at 25 µM (p=0.0409, Figure 4.2 G) and by 15% at 50 µM (p=0.0329, Figure 4.2 G).

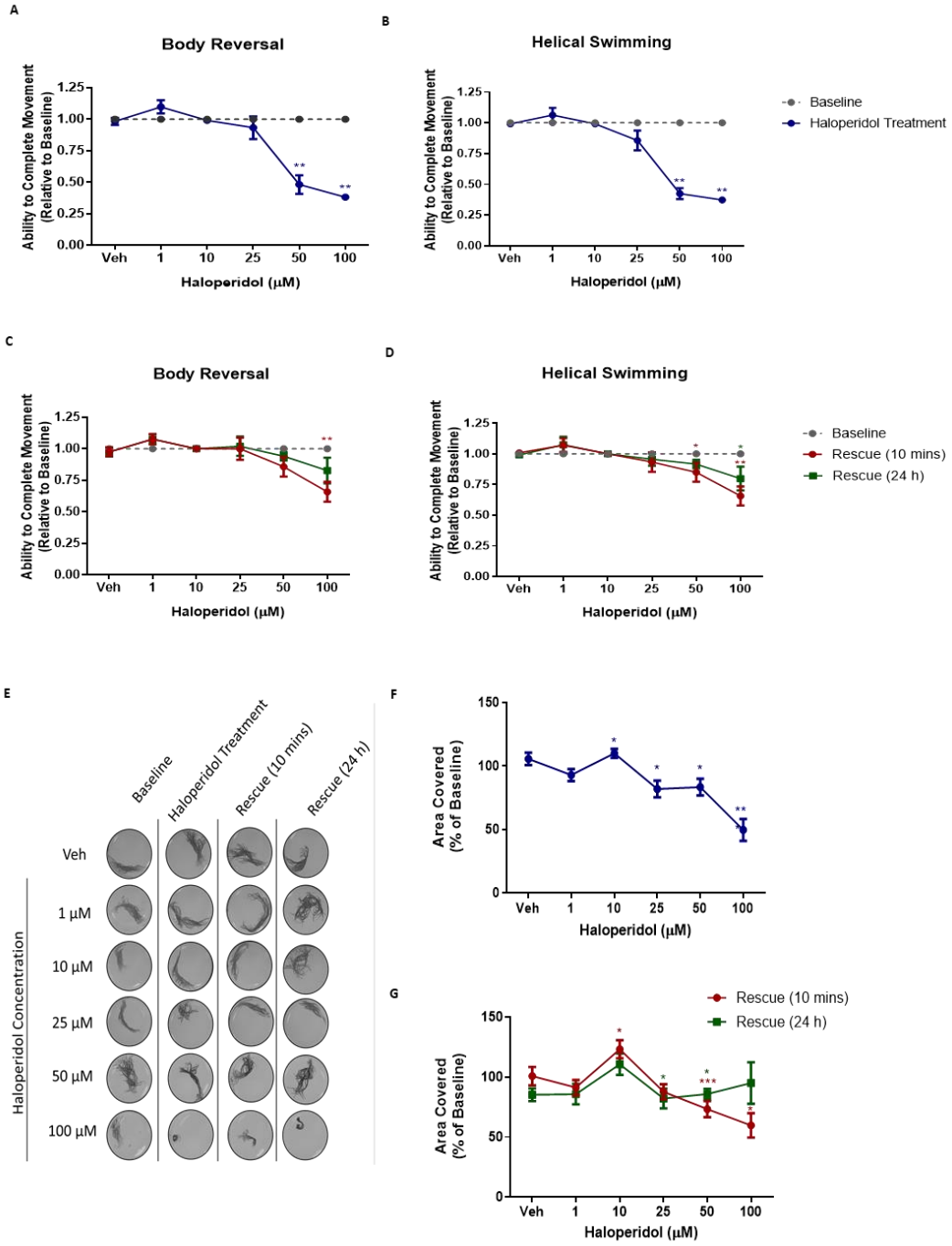

Figure 4.2. The effect of haloperidol on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of haloperidol (1 – 100 µM) and tested for the ability of tactile stimulation to elicit (A) body reversal or (B) helical swimming. Haloperidol was then removed and the ability of L. variegatus to perform (C) body reversal or (D) helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. The effect of haloperidol on free locomotion was measured before haloperidol exposure (Baseline), after 10 minutes of exposure to 1 – 100 µM haloperidol (Haloperidol Treatment), 10 minutes after haloperidol removal (Rescue (10 mins)) and 24 hours after haloperidol removal (Rescue (24 h)). Quantification of the area covered by L. variegatus following (F) haloperidol treatment and (G) removal of haloperidol for 10 minutes and 24 hours are the mean, n=8 for each concentration. Experimental repeats were conducted in collaboration with Rewash Ale. Veh: 0.5% DMSO in artificial pond water. **p*<0.05, ***p*<0.01, ***p<0.001.

To further elucidate the impact of dopaminergic signalling in this organism, we examined the effects of the selective dopamine D_2 receptor antagonist, sulpiride (Memo et al., 1981) at equimolar concentrations to haloperidol.

Unlike haloperidol, sulpiride did not affect body reversal or helical swimming ($p>0.05$, Figure 4.3 A-B). As shown in Figure 4.3 C-D, after 10-minutes and 24-hours in drug-free artificial pond water there was still no significant change and *L. variegatus* stereotypical behaviours remained at baseline levels ($p>0.05$). Much like the stereotypical behaviour assay, sulpiride had no significant effect on *L. variegatus* free locomotion ($p>0.05$, Figure 4.3 F), results remained at baseline levels after 10-minutes and 24-hours in drug-free artificial pond water ($p>0.05$, Figure 4.3 G).

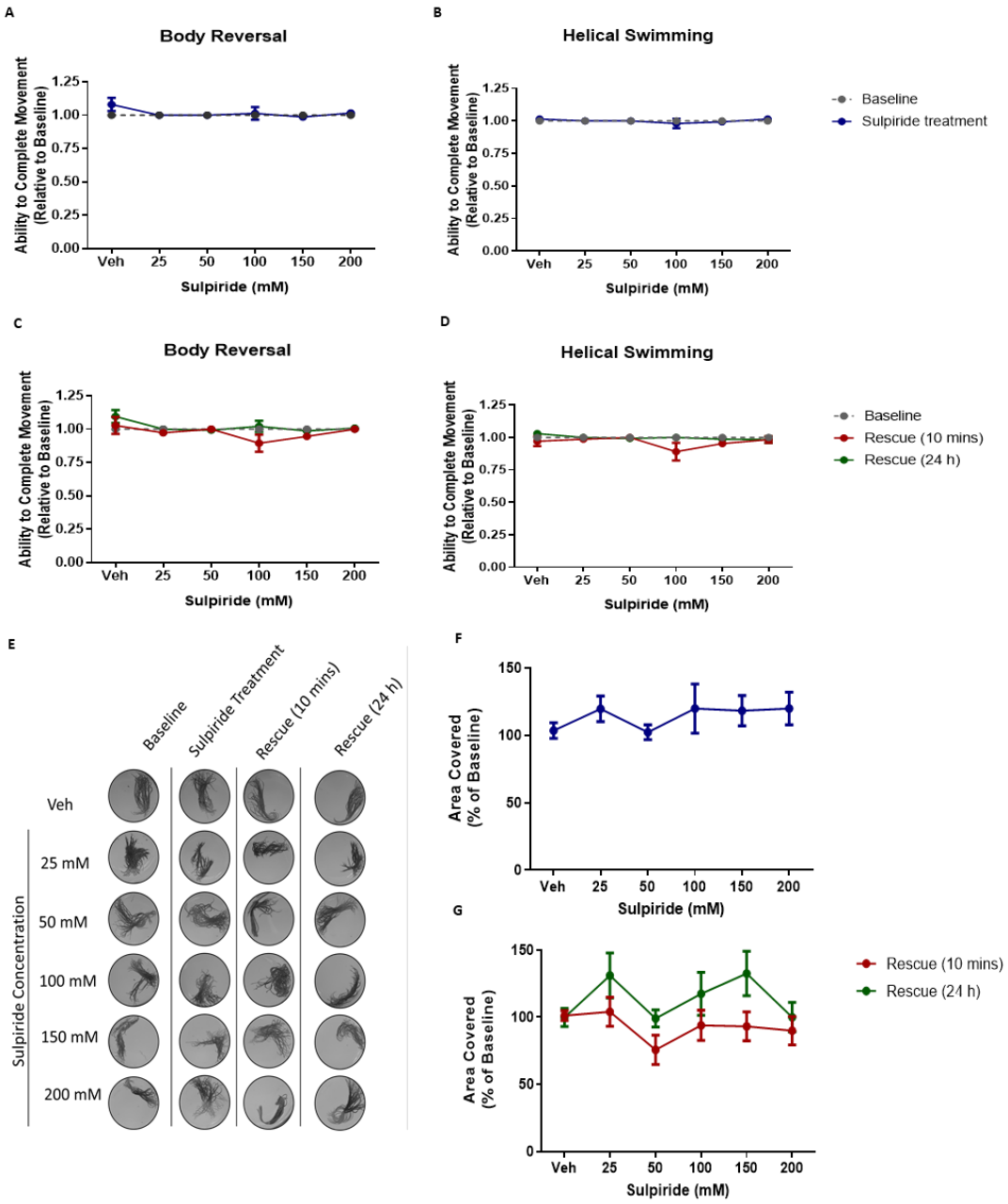

Figure 4.3. Effect of sulpiride on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of sulpiride (25 – 200 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Sulpiride was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of sulpiride on free locomotion was measured before sulpiride exposure (Baseline), after 10 minutes of exposure to 25 – 200 mM sulpiride (Sulpiride Treatment), 10 minutes after sulpiride removal (Rescue (10 mins)) and 24 hours after sulpiride removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** sulpiride treatment and **(G)** removal of sulpiride for 10 minutes and 24 hours are the mean, n=10 for each concentration. Veh: 0.5% DMSO in artificial pond water.

Having exposed *L. variegatus* to dopamine, a neurotransmitter that is both excitatory and inhibitory (Guy-Evans, 2021), we aimed to expose *L. variegatus* to a more specific neurotransmitter that interacts with ion channels.

GABA is a major inhibitory neurotransmitter that binds to post-synaptic GABA receptors which regulate ion channels (Jewett & Sharma, 2022). As such, we exposed *L. variegatus* to GABA to see whether the effects would be similar, or opposite to those seen with dopamine.

As shown in Figure 4.4 A-B, there was a decrease at 10 mM for body reversal (p<0.0078) and helical swimming (p=0.0156). Decrease in both behaviours were also seen at 50 mM (p=0.0313) and 100 mM (p=0.0078). These results persisted after 10-minutes in drug-free artificial pond water (Figure 4.4 C-D), however, after 24-hours in drug-free artificial pond water all results returned to baseline levels (p>0.05, Figure 4.4 C-D).

GABA had no significant effect on *L. variegatus* free locomotion (p>0.05, Figure 4.4 F). After 10-minutes and 24-hours in drug-free artificial pond water, the results remained the same with no significant change from baseline levels (Figure 4.4 G).

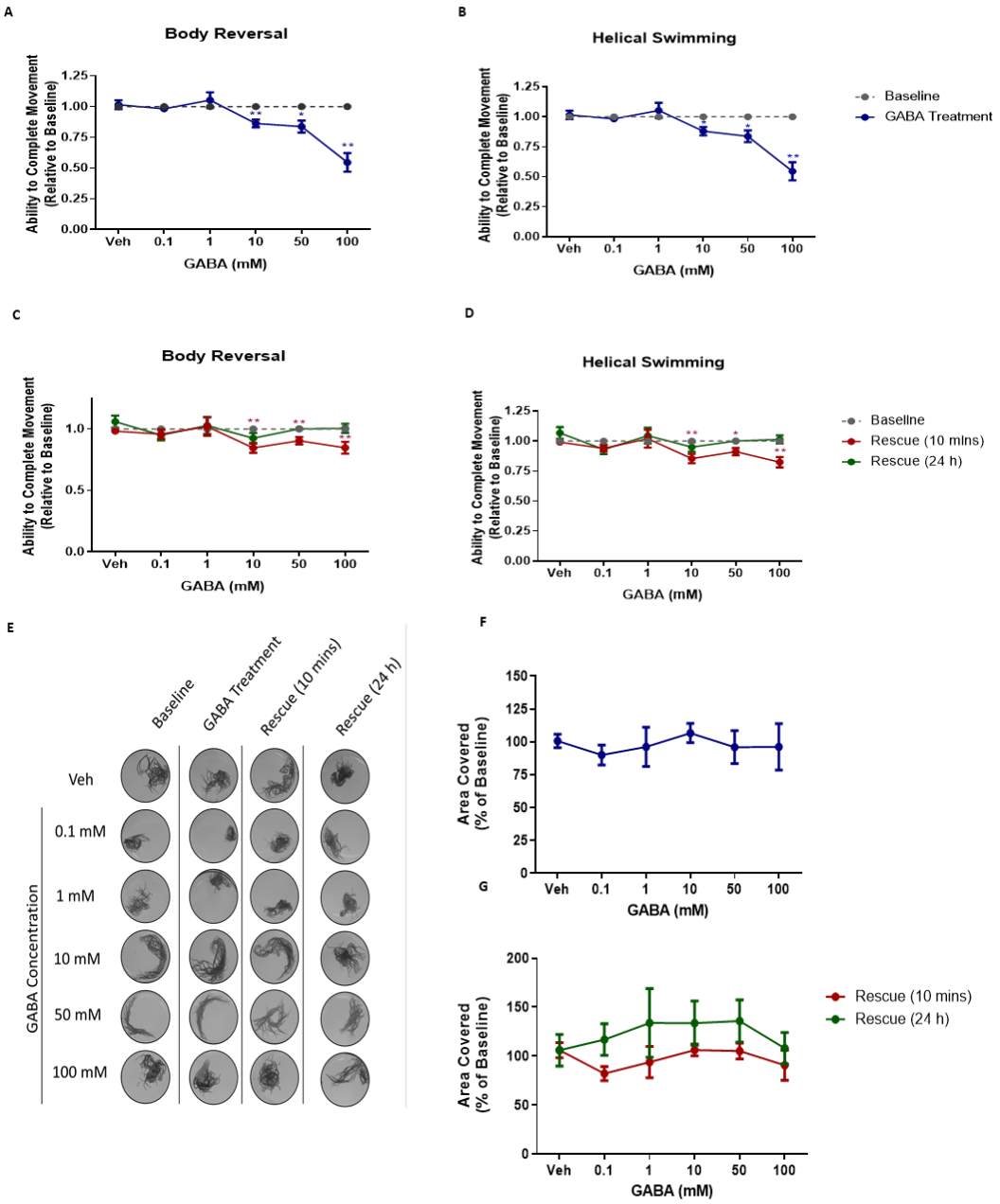

Figure 4.4. Effect of GABA on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of GABA (0.1 – 100 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. GABA was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of GABA on free locomotion was measured before GABA exposure (Baseline), after 10 minutes of exposure to 0.1 – 100 mM D (GABA Treatment), 10 minutes after GABA removal (Rescue (10 mins)) and 24 hours after GABA removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** GABA treatment and **(G)** removal of GABA for 10 minutes and 24 hours are the mean, n=8 for each concentration. Experimental repeats were conducted in collaboration with Shaurya Nathan Mathur. Veh: artificial pond water. *p<0.05, **p<0.01.

To further the investigation into whether *L. variegatus* possess neurotransmitters, we exposed them to the $GABA_A$ receptor antagonist bicuculline (Johnston, 2013). Bicuculline is shown to have antagonistic effects on GABA (Johnston, 2013), and glycine receptors containing α2 subunits (Li & Slaughter, 2007).

There was a significant decrease in *L. variegatus* stereotypical movements when exposed to ≥50 µM of bicuculline ($p<0.05$, Figure 4.6 A-B). After 10-minutes in drug-free artificial pond water, there was still body reversal and helical swimming was still inhibited compared to baseline levels at concentrations ≥50 µM ($p<0.001$, Figure 4.6 C-D), however, after 24-hours in drug-free artificial pond water, these results returned to baseline levels ($p>0.05$, Figure 4.6 C-D).

Unlike in the stereotypical behaviour assay, *L. variegatus* free locomotion was increased by 15% when exposed to 5 µM of bicuculline ($p=0.0694$, Figure 4.6 F). There was also a change in movement at 250 µM, as there was a 38% decrease ($p=0.0005$, Figure 4.6 F). However, after 10-minutes in drug-free artificial pond water, there was a 29% decrease in movement at 125 µM ($p=0.0471$, Figure 4.6 G). After 24-hours in drug-free artificial pond water all results returned to baseline levels ($p>0.05$, Figure 4.6 G)

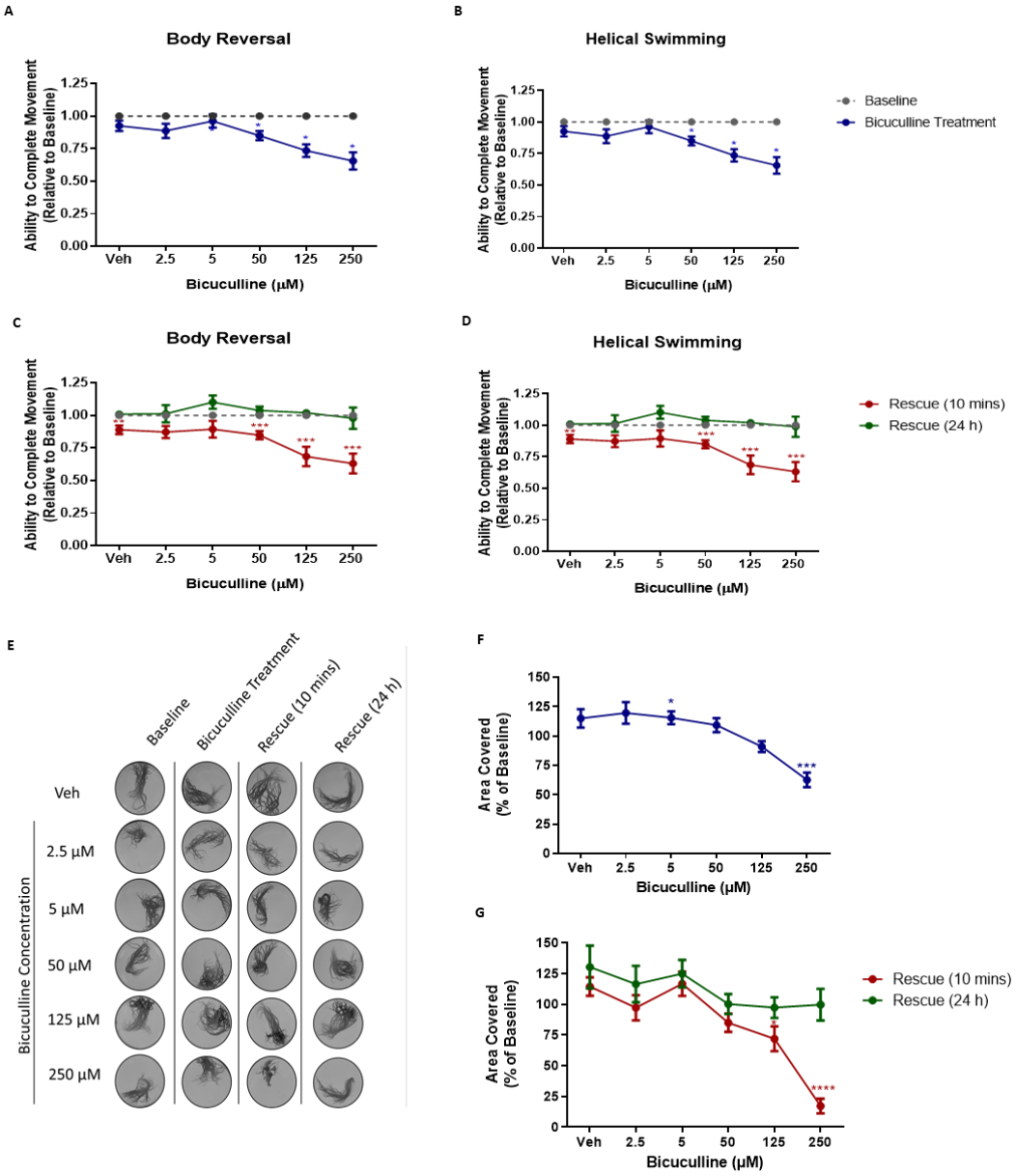

Figure 4.5. Effect of bicuculline on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of bicuculline (2.5 - 250 µM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Bicuculline was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of bicuculline on free locomotion was measured before bicuculline exposure (Baseline), after 10 minutes of exposure to 2.5 - 250 µM (bicuculline Treatment), 10 minutes after bicuculline removal (Rescue (10 mins)) and 24 hours after bicuculline removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** bicuculline treatment and **(G)** removal of bicuculline for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: 0.5% DMSO in artificial pond water. **p*<0.05, ****p*<0.001, *****p*<0.0001.

Having exposed *L. variegatus* to GABA, an inhibitory neurotransmitter, we wanted to look into the effects of other inhibitory neurotransmitters. Glycine is an inhibitory neurotransmitter (Van Den Eynden et al., 2009). In some areas of the central nervous system, glycine is co-released with the main inhibitory amino acid neurotransmitter GABA. The reuptake of glycine is a process that is activated by the electrochemical gradient of sodium ions through the plasma membrane (López-Corcuera et al., 2001).

When exposed to glycine, *L. variegatus* body reversal was inhibited at 500 mM (p=0.0078, Figure 4.5 A). However, helical swimming was inhibited at 250 mM (p=0.0313, Figure 4.5 B) and 500 mM (p=0.0078, Figure 4.5 B). These results persisted after 10-minutes in drug-free artificial pond water, with the addition of a decrease in body reversal at 250 mM (p=0.0288, Figure 4.5 C), and inhibition in body reversal and helical swimming at 500 mM (p<0.0001, Figure 4.5 C-D). After 24-hours in drug-free artificial pond water, results returned to baseline levels, except at 500 mM for both body reversal (p=0.0011, Figure 4.5 C) and helical swimming (p=0.0007, Figure 4.5 D).

Glycine significantly decreased *L. variegatus* free locomotion at 500 mM by 89% (p<0.0001, Figure 4.5 F). These results persisted after 10-minutes in drug-free artificial pond water where movement was decreased by 86% (p<0.0001, Figure 4.5 G) compared to baseline. After 24-hours in drug-free artificial pond water, movement was still decreased by 56% at 500 mM (p=0.0017, Figure 4.5 G).

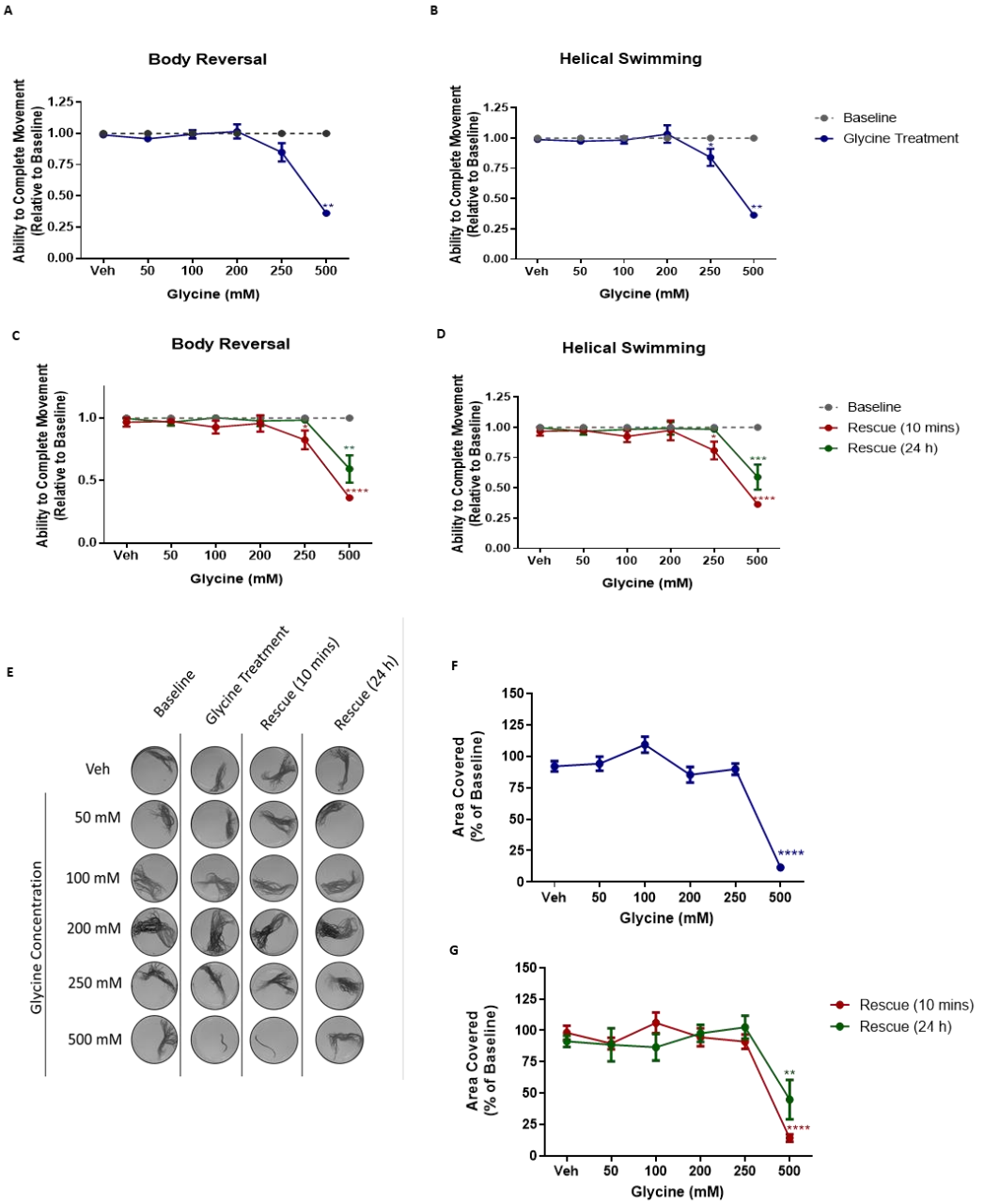

Figure 4.6. The effect of glycine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of glycine (50 – 500 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Glycine was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of glycine on free locomotion was measured before glycine exposure (Baseline), after 10 minutes of exposure to 50 – 500 mM D (Glycine Treatment), 10 minutes after glycine removal (Rescue (10 mins)) and 24 hours after glycine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** Glycine treatment and **(G)** removal of glycine for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: artificial pond water. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Next, we sought to determine the impact of exogenous compounds, therefore we exposed *L. variegatus* to ethanol. Ethanol affects ligand-gated ion channels, as well as voltage-dependent calcium channels, and is also known to potentiate GABAergic activity (Davies, 2003).

As seen in Figure 4.7 A, ethanol significantly decreased body reversal at ≥250 mM (p=0.0078). Helical swimming was also inhibited at 250 mM (p=0.0156, Figure 4.7 B) and 500 mM (p=0.0078, Figure 4.6 B). After 10-minutes and 24-hours in drug-free artificial pond water, these results returned to baseline levels (p>0.05, Figure 4.7 C-D)

Similar to stereotypical behaviour, ethanol inhibited *L. variegatus* free locomotion at concentrations ≥250 mM. Movement was decreased by 45% at 250 mM (p=0.0004, Figure 4.7 F) and 53% at 500 mM (p<0.0001, Figure 4.7 F). After 10-minutes and 24-hours in drug-free artificial pond water, these results returned to baseline levels (p>0.05, Figure 4.7 G).

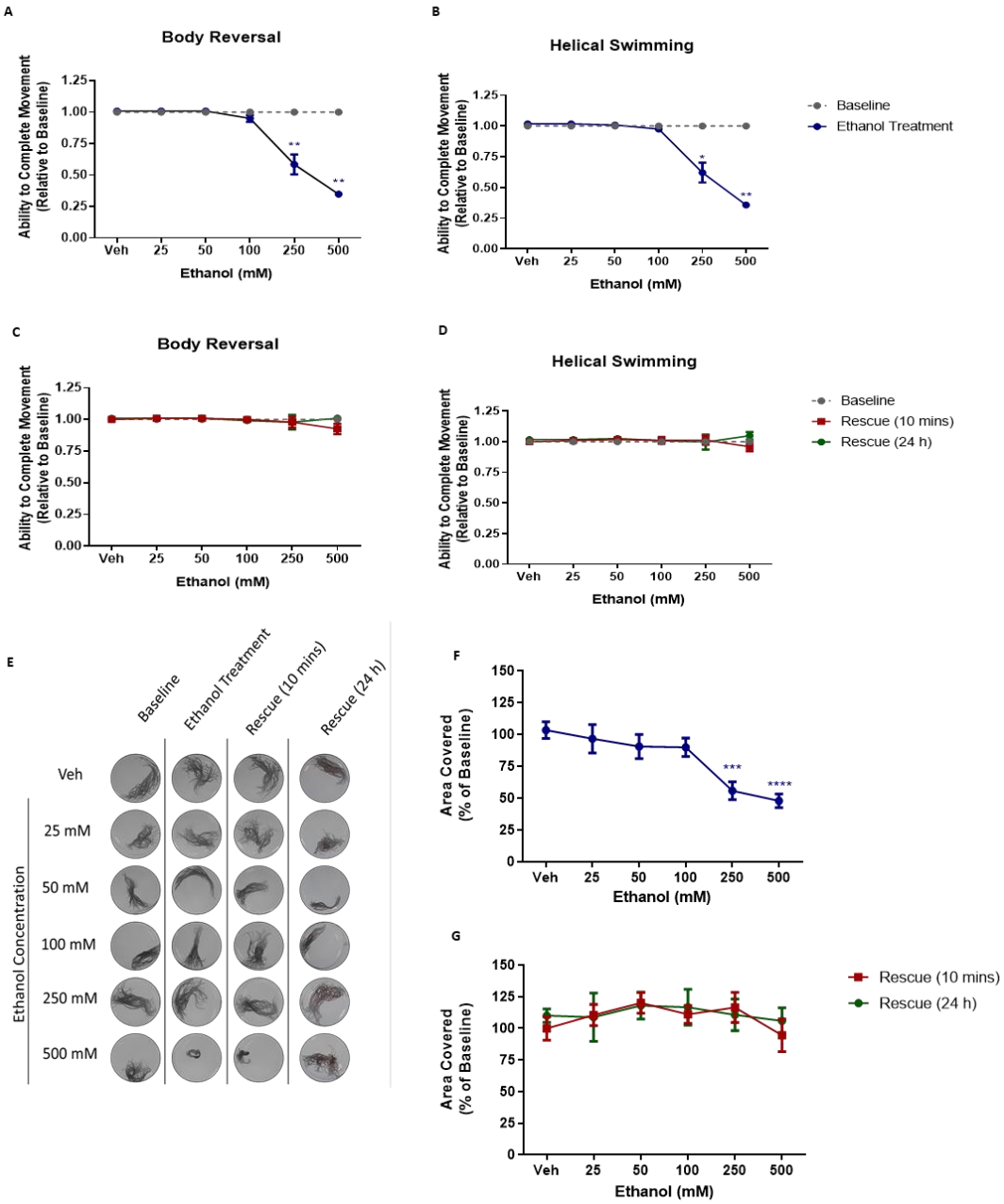

Figure 4.7. Effect of ethanol on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of ethanol (25 – 500 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Ethanol was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of ethanol on free locomotion was measured before ethanol exposure (Baseline), after 10 minutes of exposure to 25–500 mM (Ethanol Treatment), 10 minutes after ethanol removal (Rescue (10 mins)) and 24 hours after ethanol removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** Ethanol treatment and **(G)** removal of ethanol for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: artificial pond water. **p*<0.05, ***p*<0.01, ****p*<0.001,

As we had characterised a number of exogenous and endogenous compounds, to further validate *L. variegatus* as an *in vivo* model for research we sort to develop other methods and assays. Having exposed *L. variegatus* to ethanol and seeing the effect it has on their behaviours, as well as looking at existing studies with *C. elegans*, we decided to optimise an assay that looked into *L. variegatus* acute tolerance to ethanol. Acute tolerance can be defined as a form of behavioural plasticity, which can be divided into 3 categories: acute, rapid and chronic. Here we look at acute, which develops within minutes of being exposed to ethanol (Pietrzykowski & Treistman, 2008).

Figure 4.8 A shows a z-stacks of *L. variegatus* movement over 210-minutes. We chose to do this assay over 210-minutes as an existing study by Scholz et al (2000) measured the ethanol concentration in *Drosophila* at 0, 30, 60 and 210-minutes. All time points were compared with 10-minutes exposure. The area covered after 10-minutes of ethanol exposure was 33%, and as seen in Figure 4.8. B the only significant difference in movement observed was at 210-minutes, where the area covered was increased to 57% (p=0.0157).

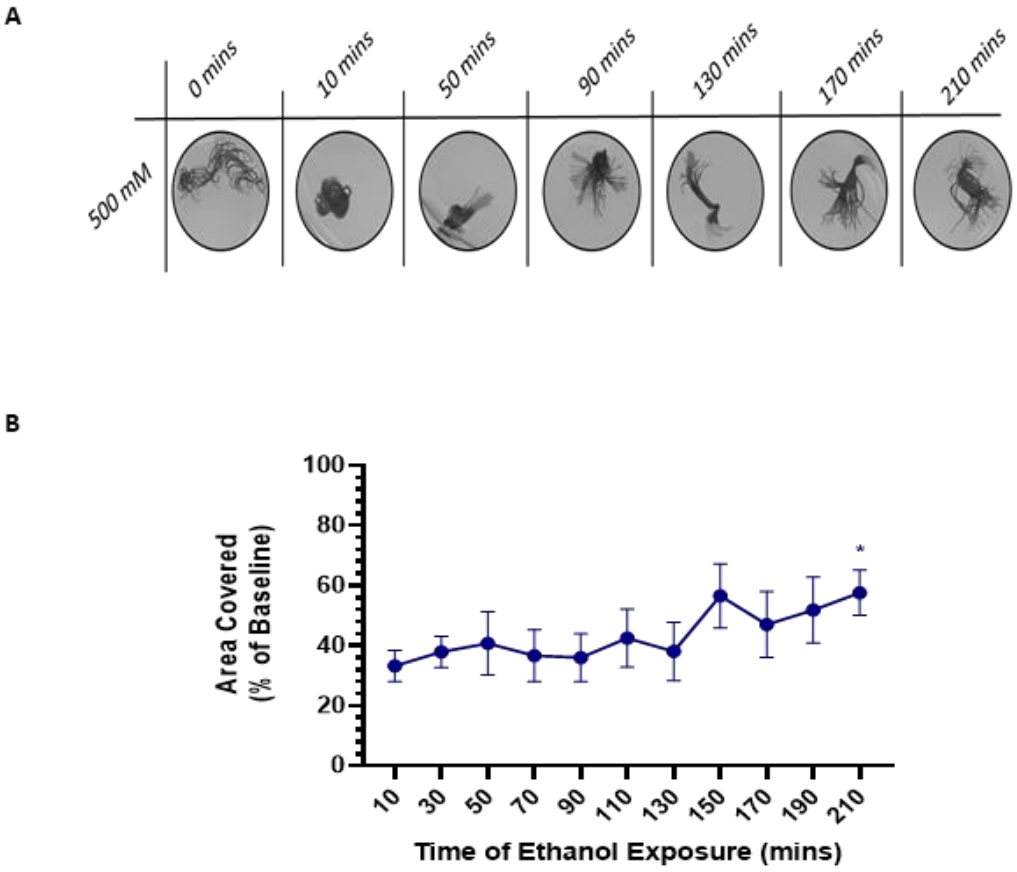

Figure 4.8. The demonstration of acute functional tolerance in *Lumbriculus variegatus* during 500mM ethanol exposure. (A) *L. variegatus* free locomotion was measured after 10 minutes of exposure to 500mM of ethanol and then at 20-minute intervals for 210 minutes. (B) Quantification of *L. variegatus* free locomotion expressed as a percentage of free locomotion before ethanol exposure. Statistical differences were measured by one-way ANOVA with Dunnett's post-test compared to the 10 minutes of ethanol exposure. Error bars represent the standard error of the mean. **p*<0.05. n=12. Experimental repeats were conducted by Julanta Carriere and Shaurya Nathan Mathur.

To further validate *L. variegatus* as an *in vivo* model we needed to look beyond their ability to perform stereotypical behaviours when exposed to exogenous compounds and look at the model on a molecular scale.

A review by Acosta et al (2021) talks about the molecular processes of regeneration in *L. variegatus*. The review also discusses the work of Tellez-Garcia et al (2021), who conducted a transcriptome analysis during the early regeneration of *L. variegatus* using RNA sequencing. This was the first regeneration transcriptome to be done for *L. variegatus*, and 136 differentially expressed transcripts were identified during early regeneration (Tellez-Garcia et al., 2021). Of the 136 transcripts identified, 73 were potentially protein-coding and had BLASTp hits known to certain proteins, which were all genes found to be differentially expressed during the regeneration process of annelids (Acosta et al., 2021).

Before extracting protein from *L. variegatus*, we first exposed them to ethanol, as the previous ethanol data from this study was clear. Ethanol was used as it is known to rapidly permeate cells (Patra et al., 2006) and is readily available within the lab.

As shown in Figure 4.9 A, a standard curve was made using BSA as a protein standard via the Bradford assay. Samples were subjected to SDS-PAGE and gels containing proteins were stained with Coomassie blue (Figure 4.9 B) or transferred to PVDF membrane and stained using Ponceau (Figure 4.9 C).

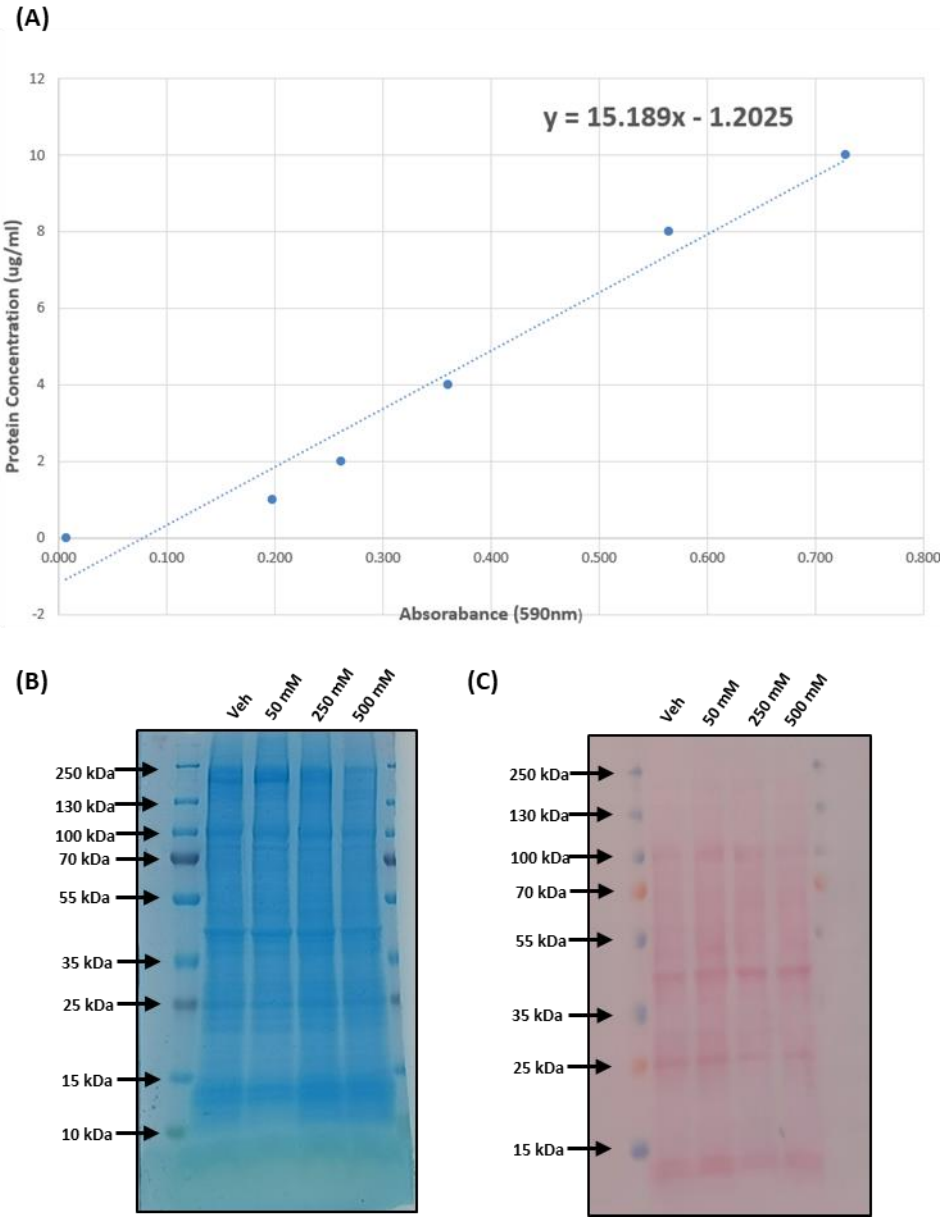

Figure 4.9. Protein quantification of *Lumbriculus variegatus* samples after exposure to ethanol. *L. variegatus* were exposed to increasing concentrations of ethanol (50 - 500mM) for 10 minutes. After being removed from ethanol and rinsed with artificial pond water, L. variegatus were homogenised and protein was extracted. **(A)** Protein samples were quantified according by the Bradford assay (Bradford, 1976) and a standard curve with bovine serum albumin (BSA) was used to calculate protein concentrations in *L. variegatus*. Proteins were separated by performing SDS-PAGE using polyacrylamide gel. PageRuler Plus Stained Protein Ladder was used as a reference for protein size. **(B)** Gel containing proteins was stained with Coomassie blue or **(C)** membrane proteins transferred onto PVDF membrane and stained using Ponceau S. n=1 for each concentration. Veh: artificial pond water.

As we successfully purified protein, we sort out to extract and quantify *L. variegatus* DNA. We based our method for this assay on a paper by Gustafsson et al, 2008 who successfully extracted and amplified *L. variegatus* DNA.

Similar to the protein extraction, we exposed *L. variegatus* to ethanol prior to DNA extraction and quantification. We then extracted L. variegatus DNA using E.N.Z.A.® Tissue DNA Kit (Omega Bio-Tek) and purified samples were quantified using a Nanodrop spectrophotometer.

Table 4.1 shows the concentration of ethanol each sample was exposed to and the concentration of DNA present in each sample.

Table 4.1. Quantification of DNA extracted from *Lumbriculus variegatus* after ethanol exposure.

Sample	Concentration (µg/µL)	260:280	260:230
Veh	133.0	2.0	2.4
25 mM	108.9	1.9	2.0
50 mM	44.2	1.9	2.2
100 mM	41.4	1.9	2.4
250 mM	72.2	1.9	2.3
500 mM	43.0	1.9	2.4

4.1. Chapter Summary

In this section, we exposed *L. variegatus* to several different neurotransmitters and exogenous compounds that block or potentiate their activity. We also successfully extracted and quantified *L. variegatus* protein, as well as extracted and amplified DNA.

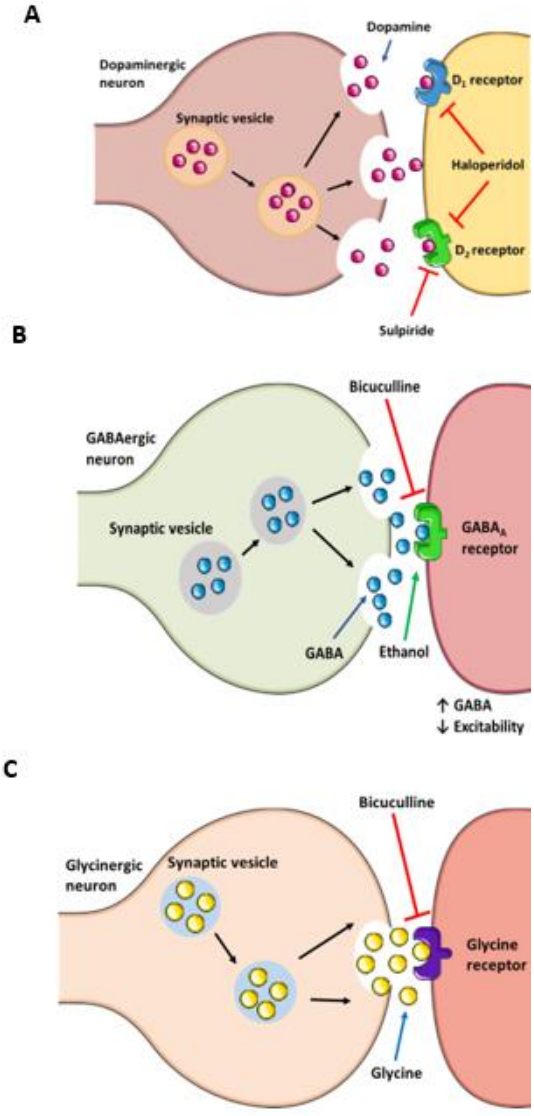

Figure 4.10. Schematic of antagonists on excitatory and inhibitory neurotransmission in humans. Figure 4.10 A shows the inhibition of excitatory neurotransmission. The non-specific dopamine receptor antagonist haloperidol, binds to dopamine D1/D2 receptors, and selective dopamine receptor antagonist sulpiride binds to D_2 receptor, both blocking dopaminergic neurotransmission. Figure 4.10 B shows the blocking of inhibitory neurotransmission. Bicuculline acts as a competitive antagonist at GABAa binding sites, blocking GABAergic neurotransmission, whereas ethanol potentiates GABA, leading to a decrease in excitability. Figure 4.10 C shows another example of inhibition in inhibitory neurotransmission. Bicuculline binds to glycine receptors, predominantly at GlyRα2 receptors, stopping glycinergic neurotransmission.

5. Results chapter 3: Investigating the effects of 2,4-dinitrophenol and potential antidote therapies in *L. variegatus*

To further validate the applicability of *L. variegatus* for use in pharmacology and toxicology research we determined whether *L. variegatus* can recover when exposed to a toxic substance, followed by a potential antidote therapy.

Here we expose *L. variegatus* to increasing concentrations of 2,4-dinitrophenol (DNP), a toxic uncoupler of oxidative phosphorylation, that can cause hyperthermia and death (Grundlingh et al., 2011a).

As seen in Figure 5.1 A-B, DNP inhibits *L. variegatus* stereotypical behaviours at 5 µM (p=0.0156) and ≥12.5 µM (p=0.0078). After 10-minutes in drug-free artificial pond water, the effects at 5 µM persist for both body reversal (p=0.0139, Figure 5.1 C) and helical swimming (p=0.0499, Figure 5.1 D). A decrease in behaviours compared to baseline is still seen at ≥12.5 µM (p<0.0001, Figure 5.1 C-D) after 10-minutes in drug-free artificial pond water. After 24-hours in drug-free artificial pond water, all results return to baseline levels, with the exception of 25 µM (p<0.01, Figure 5.1 C-D) and 50 µM (p<0.0001, Figure 5.1 C-D).

As seen with the stereotypical behaviours, DNP inhibits *L. variegatus* free locomotion at ≥5 µM (Figure 5.1 F). Movement is decreased by 20% at 5 µM (p=0.0490, Figure 5.1 F) and by 80% at 50 µM (p<0.0001, Figure 5.1 F). After 10-minutes in drug-free artificial pond water, these effects are still seen at concentrations ≥12.5 µM (Figure 5.1 G). After 24-hours in drug-free artificial pond water, changes seen at concentrations ≤12.5 µM return to baseline levels (p>0.05, Figure 5.1 G). However, there is still a 27% decrease in movement at 25 µM (p=0.0186, Figure 5.1 G) and 77% decrease at 50 µM (p<0.0001, Figure 5.1 G).

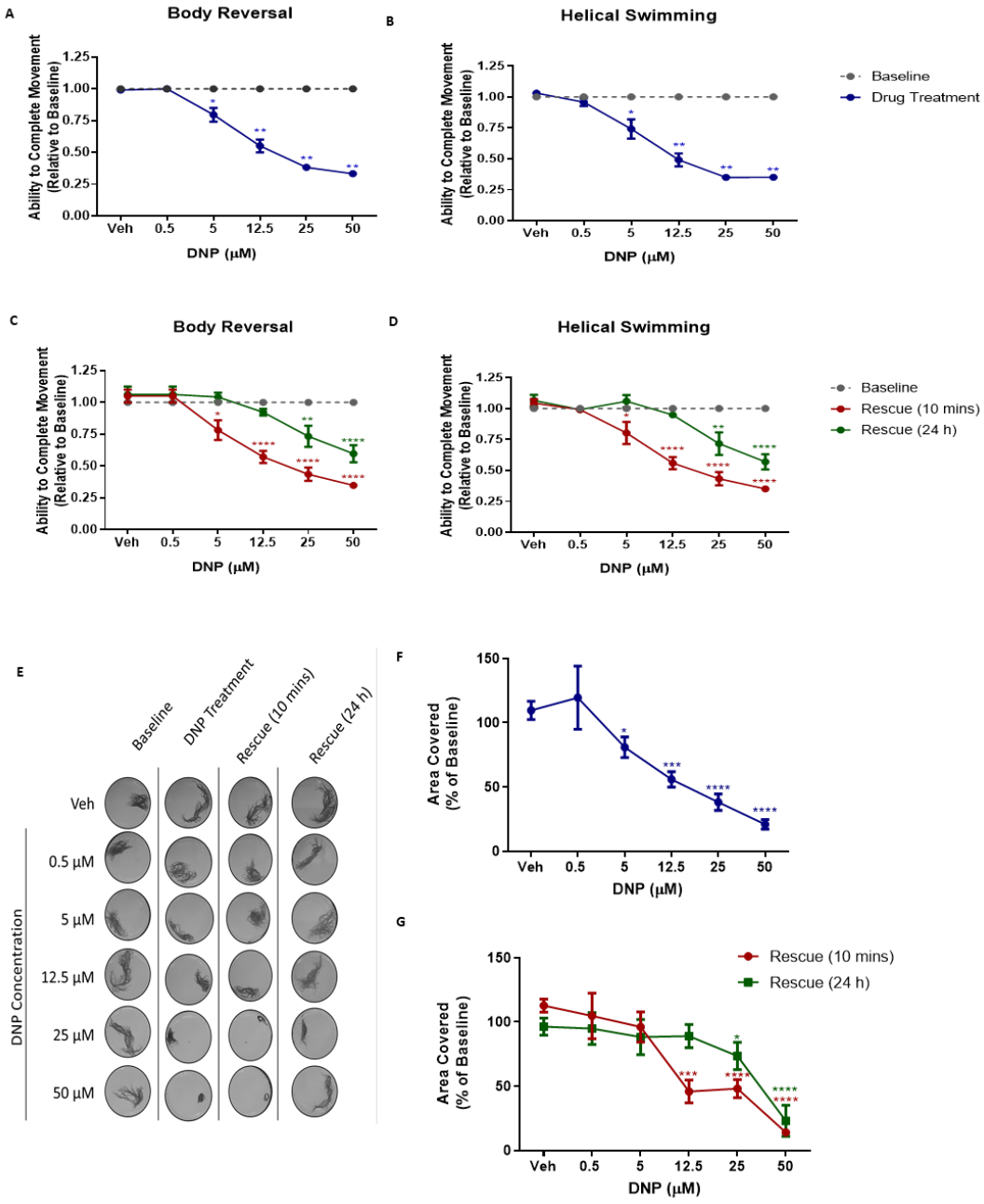

Figure 5.1. The effect of 2,4-dinitrophenol on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of DNP (0.5 – 50 µM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. DNP was then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of DNP on free locomotion was measured before DNP exposure (Baseline), after 10 minutes of exposure to 0.5 – 50 µM DNP (DNP Treatment), 10 minutes after DNP removal (Rescue (10 mins)) and 24 hours after DNP removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** DNP treatment and **(G)** removal of DNP for 10 minutes and 24 hours are the mean, n=8 for each concentration. Experimental repeats were conducted in collaboration with Yusuf Hussein, Rewash Ale, Beatrix Banka and Jonah Moxey. Veh: 0.5% DMSO in artificial pond water. **p*<0.05,

Dantrolene is used in the treatment of malignant hyperthermia (Krause et al., 2004), and has been used clinically for treating DNP poisoning (Kopec et al., 2019). As DNP affected *L. variegatus* stereotypical behaviours and free locomotion, we moved on to DNP exposure, followed by dantrolene. As we have previously exposed *L. variegatus* to dantrolene, we were able to determine the no observable adverse effect level (NOAEL) as 12.5 µM. As such, we exposed *L. variegatus* to the same increasing concentrations of DNP followed by 12.5 µM of dantrolene, to see whether the effects of DNP could be reversed.

Similar to what is seen in Figure 5.1 A-B, when exposed to DNP and dantrolene in combination there is a decrease in *L. variegatus* stereotypical behaviours at concentrations ≥5 µM (Figure 5.2 A-B). After 10-minutes in drug-free artificial pond water, the effects seen at 5 µM persist for body reversal (p=0.0435, Figure 5.2 C) and helical swimming (p=0.0002, Figure 5.2 D). At concentrations ≥12.5 µM, there is still a decrease compared to baseline levels for both body reversal and helical swimming (p<0.0001, Figure 5.2 C-D). After 24-hours in drug-free artificial pond water, all results return to baseline levels, except for at 50 µM where a significant decrease is seen in both body reversal (p=0.0008, Figure 5.2 C) and helical swimming (p=0.0003, Figure 5.2 D).

Unlike the stereotypical movements, a decrease in *L. variegatus* free locomotion was only seen at 50 µM (p=0.0003, Figure 5.2 F). At this concentration, movement was decreased by 70%. After 10-minutes in drug-free artificial pond water, these results persisted as movement was still decreased by 54% at 50 µM (p=0.0023, Figure 5.2 G). After 24-hours in drug-free artificial pond water, all results returned to baseline levels (p>0.05, Figure 5.2 G).

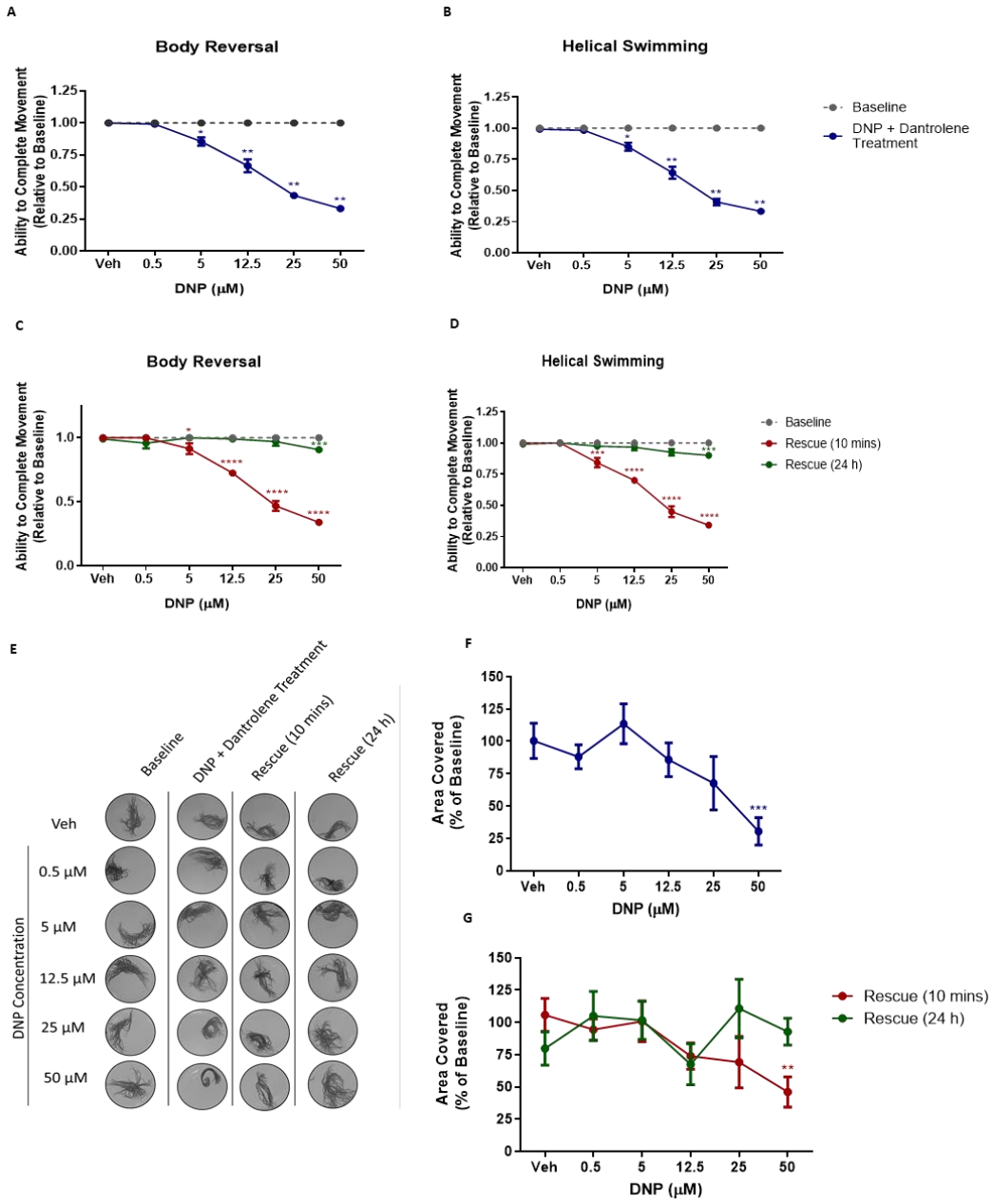

Figure 5.2. The effect of DNP followed by 12.5 µM dantrolene on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of DNP (0.5 – 50 µM) followed by 12.5 µM of dantrolene and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. DNP and dantrolene were then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of DNP and dantrolene in combination on free locomotion was measured before DNP and dantrolene exposure (Baseline), after 10 minutes of exposure to 0.5 – 50 µM DNP and 12.5 µM dantrolene (DNP + Dantrolene Treatment), 10 minutes after DNP and dantrolene removal (Rescue (10 mins)) and 24 hours after DNP and dantrolene removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** DNP + dantrolene treatment and **(G)** removal of DNP and dantrolene for 10 minutes and 24 hours are the mean, n=8 for each concentration. Experimental repeats were conducted in collaboration with Yusuf Hussein and Beatrix Banka. Veh: 0.5% DMSO in artificial pond water. **p*<0.5, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Plater & Harrison (2019) demonstrated that quinine could potentially lower the effects of DNP toxicity by forming an acid-base complex with DNP. Although quinine has not been used clinically to treat DNP poisoning, as we have previously exposed *L. variegatus* to it we had determined the NOAEL as 0.1 mM. Here we exposed *L. variegatus* to increasing concentrations of DNP followed by 0.1 mM of quinine.

When exposed to DNP and quinine in combination for 10-minutes, no significant changes were observed in *L. variegatus* stereotypical behaviours compared to baseline levels ($p>0.05$, Figure 5.3 A-B). However, after 10-minutes in drug-free artificial pond water, a decrease was seen in body reversal at 25 µM ($p=0.003$, Figure 5.3 C) and 50 µM ($p=0.0228$, Figure 5.3 C). Figure 5.3 D shows that after 10-minutes in drug-free artificial pond water there is a decrease in helical swimming at concentrations of 5 µM ($p=0.0217$), 12.5 µM ($p=0.0002$), 25 µM ($p=0.0123$) and 50 µM ($p=0.0005$). After 24-hours in drug-free artificial pond water, all results returned to baseline level ($p>0.05$, Figure 5.3 C-D).

Figure 5.3 F shows there was a decrease in *L. variegatus* free locomotion at 25 µM as movement was reduced by 84% ($p=0.0007$). There was also a 66% decrease seen at 50 µM ($p=0.0220$). These results persisted after 10-minutes in drug-free artificial pond water as movement was decreased by 88% at 25 µM ($p=0.0081$, Figure 5.3 G) and 69% at 50 µM ($p=0.0189$, Figure 5.3 G). After 24-hours in drug-free artificial pond water these results returned to baseline level ($p>0.05$, Figure 5.3 G).

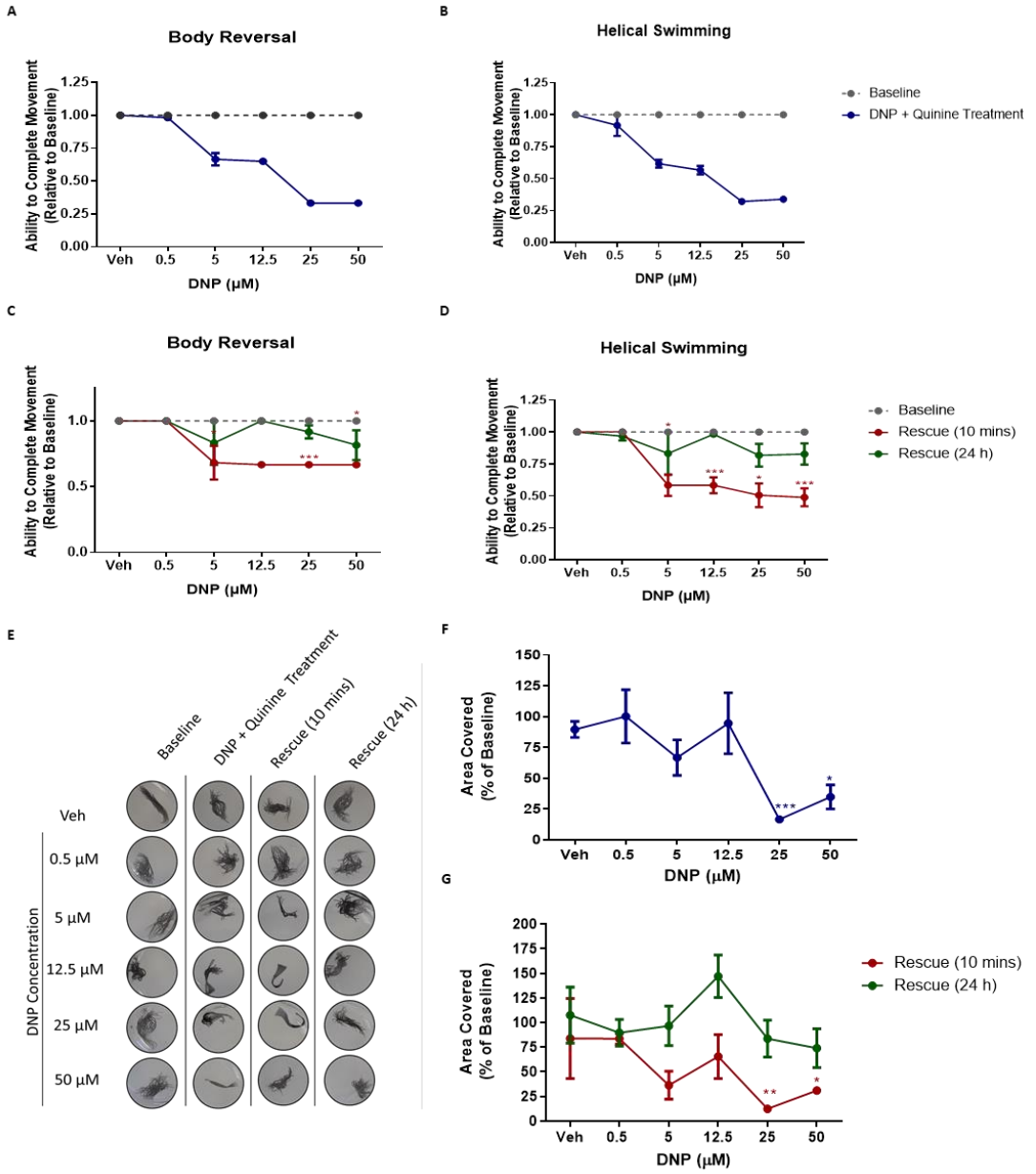

Figure 5.3. The effect of 2,4-dinitrophenol followed by 0.1 mM of quinine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of DNP (0.5 – 50 µM) followed by 0.1 mM of quinine and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. DNP and quinine were then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of DNP and quinine in combination on free locomotion was measured before DNP and quinine exposure (Baseline), after 10 minutes of exposure to 0.5 – 50 µM DNP and 0.1 mM quinine (DNP + quinine Treatment), 10 minutes after DNP and quinine removal (Rescue (10 mins)) and 24 hours after DNP and quinine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** DNP + quinine treatment and **(G)** removal of DNP and quinine for 10 minutes and 24 hours are the mean, n=4 for each concentration for body reversal and helical swimming, n=3 for each concentration for free locomotion. Experimental repeats were conducted in collaboration with Jonah Moxey. Veh: 0.5% DMSO in artificial pond water. **p<0.01, ***p<0.001.

Plater and Harrison (2019), also described haloperidol as one of the compounds that potentially could form an acid-base complex with DNP, reducing the effects of toxicity.

As we have previously exposed *L. variegatus* to haloperidol, we were able to determine the NOAEL and lowest observable adverse effect level (LOAEL). Here we exposed *L. variegatus* to increasing concentrations of DNP followed by the LOAEL of haloperidol which we determined as 10 µM. This concentration caused excitatory effects in *L. variegatus*.

When exposed to increasing concentrations of DNP and 10 µM of haloperidol in combination, there was a decrease in body reversal and helical swimming at concentrations ≥12.5 µM (p<0.01, Figure 5.4 A-B). These results persisted at the same concentrations after 10-minutes in drug-free artificial pond water (p<0.0001, Figure 5.4 C-D). After 24-hours in drug-free artificial pond water, all results returned to baseline levels (p>0.05, Figure 5.4 C-D).

When observing *L. variegatus* free locomotion after exposure to DNP and 10 µM of haloperidol, movement was decreased by 43% at 25 µM (p=0.0041, Figure 5.4 F) and 71% at 50 µM (p<0.0001, Figure 5.4 F). After 10-minutes in drug-free artificial pond water movement was decreased at 25 µM (p=0.0006, Figure 5.4 G) by 62% and at 50 µM movement was decreased by 72% (p=0.0020, Figure 5.4 G). After 24-hours in drug-free artificial pond water all results returned to baseline levels (p>0.05, Figure 5.4 G).

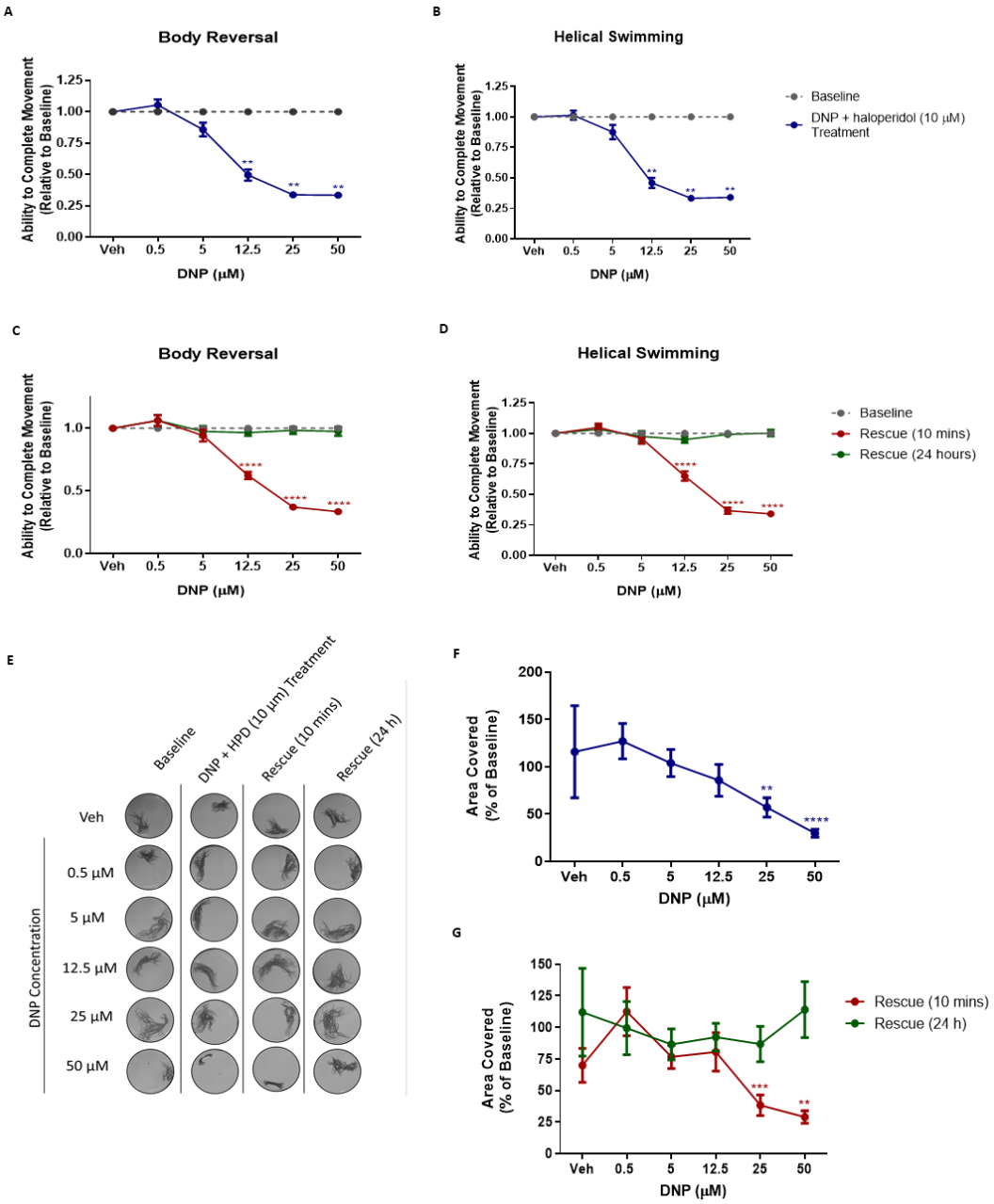

Figure 5.4. The effect of 2,4-dinitrophenol followed by 10 µM of haloperidol on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of DNP (0.5 – 50 µM) followed by 10 µM of haloperidol and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. DNP and haloperidol were then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of DNP and haloperidol in combination on free locomotion was measured before DNP and haloperidol exposure (Baseline), after 10 minutes of exposure to 0.5 – 50 µM DNP and 10 µM haloperidol (DNP + Haloperidol Treatment), 10 minutes after DNP and haloperidol removal (Rescue (10 mins)) and 24 hours after DNP and haloperidol removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** DNP + haloperidol treatment and **(G)** removal of DNP and haloperidol for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: 0.5% DMSO in artificial pond water. **p<0.01, ***p<0.001, ****p<0.0001.

As 10 µM of haloperidol successfully reversed the toxic effects of DNP after 24-hours (Figure 5.4), we exposed *L. variegatus* to increasing concentrations of DNP followed by 25 µM of haloperidol, which is what we determined as the LOAEL that causes inhibitory effects.

When exposed to DNP followed by 25 µM of haloperidol there is a decrease in *L. variegatus* body reversal and helical swimming at 5 µM (p=0.0156, Figure 5.5 A-B) and at concentrations ≥12.5 µM (p<0.01, Figure 5.5 A-B). A decrease in movement is still seen after 10-minutes in drug-free artificial pond water at ≥12.5 µM (p<0.0001, Figure 5.5 C-D). After 24-hours in drug-free artificial pond water, all results return to baseline levels (p>0.05, Figure 5.5 C-D).

Figure 5.5 F shows a decrease in *L. variegatus* free locomotion at 12.5 µM by 53% (p=0.0030) and at 25 µM by 53% (p=0.0037). Movement was also decreased at 50 µM by 80% (p<0.0001, Figure 5.5 F). After 10-minutes in drug-free artificial pond water free locomotion was decreased by 63% at 12.5 µM (p<0.0001, Figure 5.5 G), by 71% at 25 µM (p=0.0007, Figure 5.5 G) and by 66% at 50 µM (p=0.0007, Figure 5.5 G). After 24-hours in drug-free artificial pond water all results returned to baseline levels (p>0.05, Figure 5.5 G).

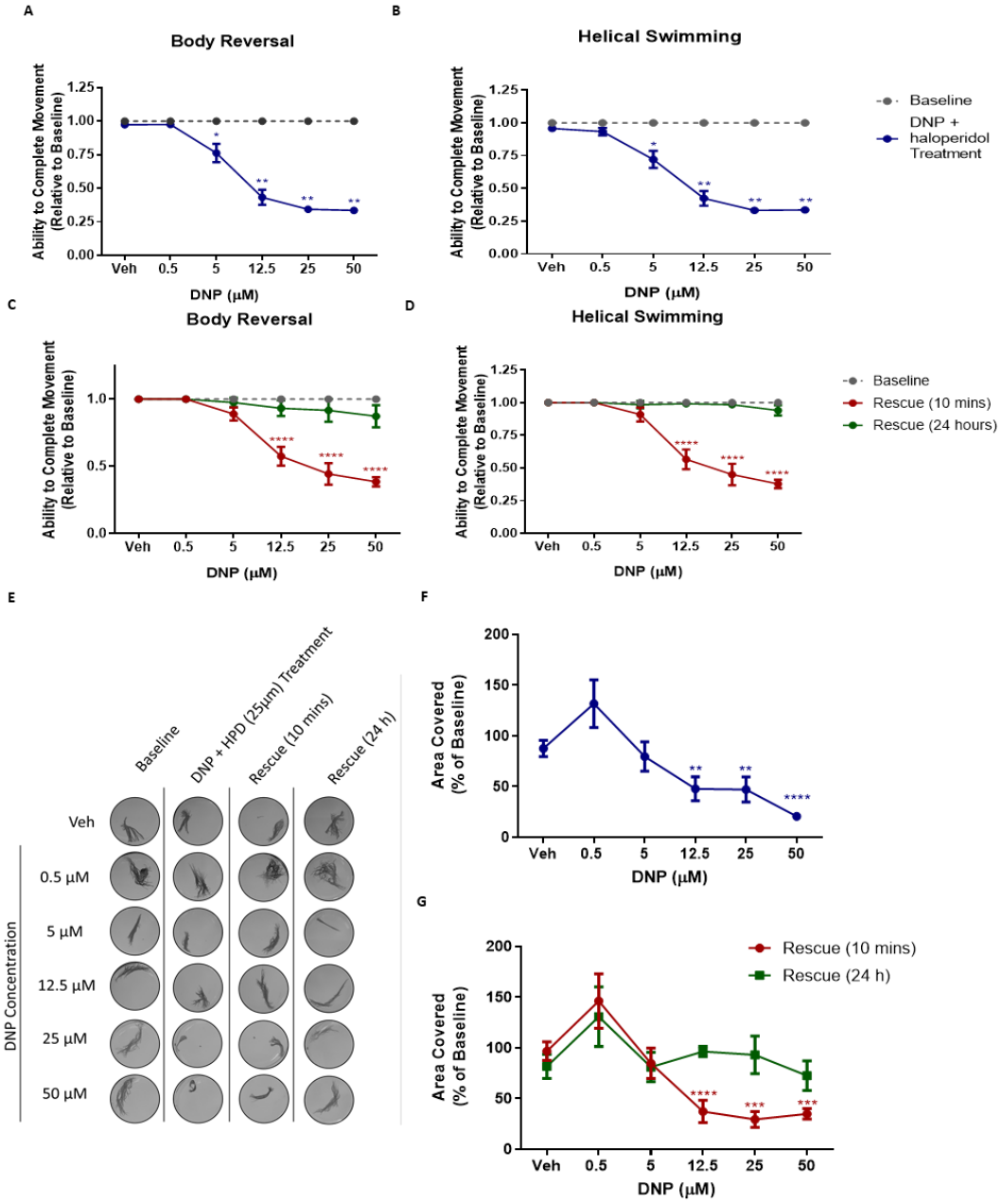

Figure 5.5. The effect of 2,4-dinitrophenol followed by 25 µM of haloperidol on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of DNP (0.5 – 50 µM) followed by 25 µM of haloperidol and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. DNP and haloperidol were then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of DNP and haloperidol in combination on free locomotion was measured before DNP and haloperidol exposure (Baseline), after 10 minutes of exposure to 0.5 – 50 µM DNP and 25 µM haloperidol (DNP + Haloperidol Treatment), 10 minutes after DNP and haloperidol removal (Rescue (10 mins)) and 24 hours after DNP and haloperidol removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** DNP + haloperidol treatment and **(G)** removal of DNP and haloperidol for 10 minutes and 24 hours are the mean, n=8 for each concentration. Experimental repeats were conducted in collaboration with Rewash Ale. Veh: 0.5% DMSO in artificial pond water. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

As haloperidol successfully relieved the symptoms of DNP toxicity in *L. variegatus*, we wanted to see whether these effects were chemically mediated, as described by Plater & Harrison, 2019, or receptor-mediated.

As we have previously exposed *L. variegatus* to sulpiride, and it is a dopamine D_2 receptor antagonist (Memo et al., 1981), we moved forward exposing *L. variegatus* to DNP in combination with sulpiride.

As sulpiride did not have any effect on *L. variegatus*, we were unable to determine the LOAEL. We decided to use 25 µM of sulpiride, as this was equimolar to the LOAEL for haloperidol.

Figure 5.6 A-B shows a significant decrease in *L. variegatus* body reversal and helical swimming at 5 µM (p=0.0313) and concentrations ≥12.5 µM (p=0.0078). This inhibition in body reversal and helical swimming persisted after 10-minutes in drug-free artificial pond water at concentrations ≥12.5 µM (p<0.0001, Figure 5.6 C-D). After 24-hours in drug-free artificial pond water all results returned to baseline level (p>0.05, Figure 5.6 C-D).

After exposure to DNP in combination with sulpiride, *L. variegatus* free locomotion was decreased by 47% at 25 µM (p=0.0002, Figure 5.6 F) and 57% at 50 µM (p=0.0004, Figure 5.6 F). After 10-minutes in drug-free artificial pond water, a 16% increase in movement was observed. There was also a further decrease in movement compared to baseline levels by 58% at 25 µM (p<0.0001, Figure 5.6 G) and by 60% at 50 µM (p<0.0001, Figure 5.6 G). After 24-hours in drug-free artificial pond water all results returned to baseline level (p>0.05, Figure 5.6 G).

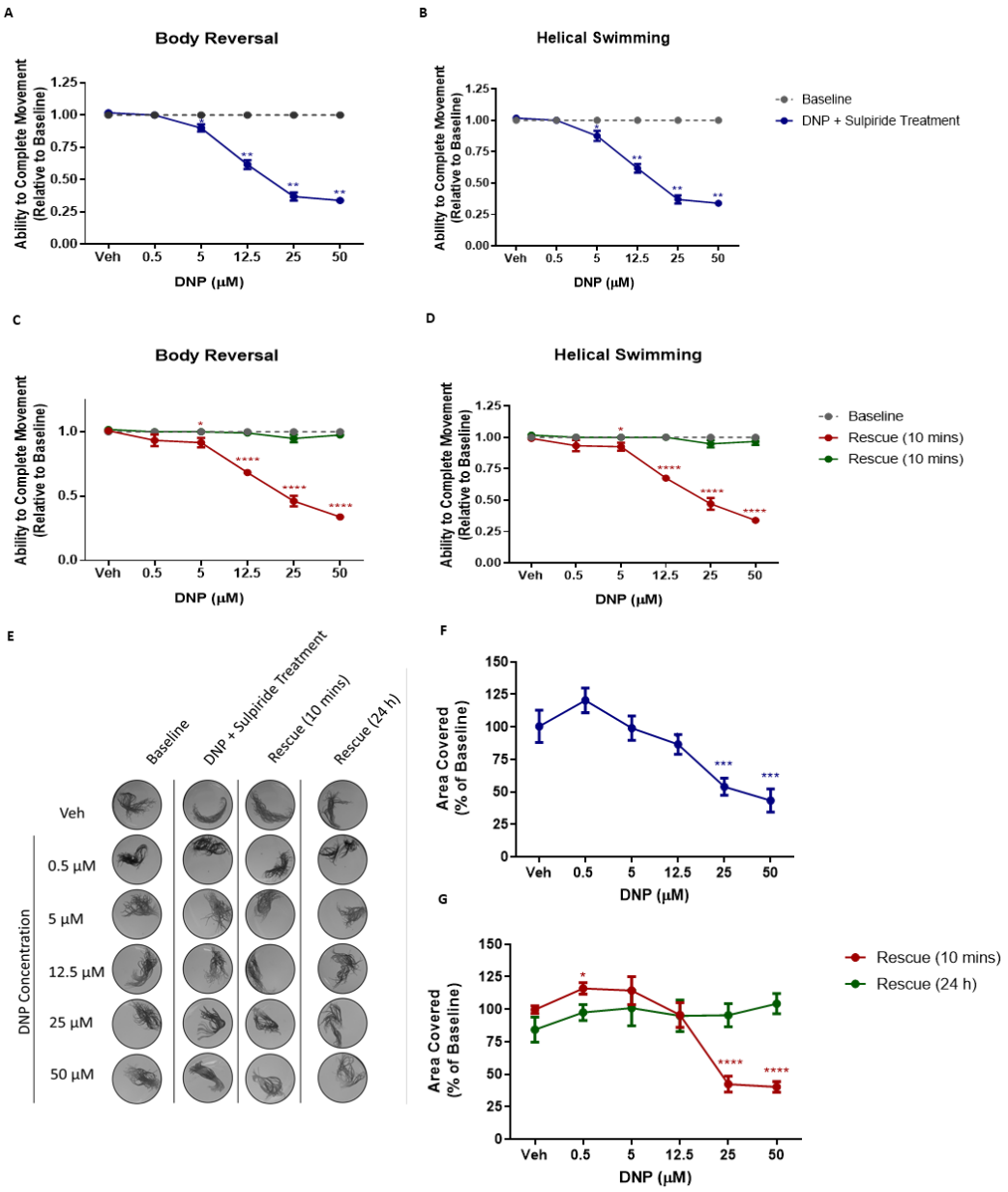

Figure 5.6. Effect of DNP followed by 25 µM sulpiride on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of DNP (0.5 – 50 µM) followed by 25 µM of sulpiride and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. DNP and sulpiride were then removed and the ability of *L. variegatus* to perform **(C)** body reversal or **(D)** helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. **(E)** The effect of DNP and sulpiride in combination on free locomotion was measured before DNP and sulpiride exposure (Baseline), after 10 minutes of exposure to 0.5 – 50 µM DNP and 25 µM sulpiride (DNP + sulpiride Treatment), 10 minutes after DNP and sulpiride removal (Rescue (10 mins)) and 24 hours after DNP and sulpiride removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** DNP + sulpiride treatment and **(G)** removal of DNP and sulpiride for 10 minutes and 24 hours are the mean, n=8 for each concentration. Veh: 0.5% DMSO in artificial pond water. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001

5.1. Chapter Summary

In this chapter we exposed *L. variegatus* to DNP alone, and DNP in combination with potential antidote therapies. Following on from a study by Plater & Harrison (2019), we exposed *L. variegatus* to DNP followed by haloperidol, which successfully reversed the toxic effects of DNP seen at 50 µM. To determine whether this response was chemical or receptor mediated, we also exposed *L. variegatus* to sulpiride, which like haloperidol reversed the toxic effects of DNP.

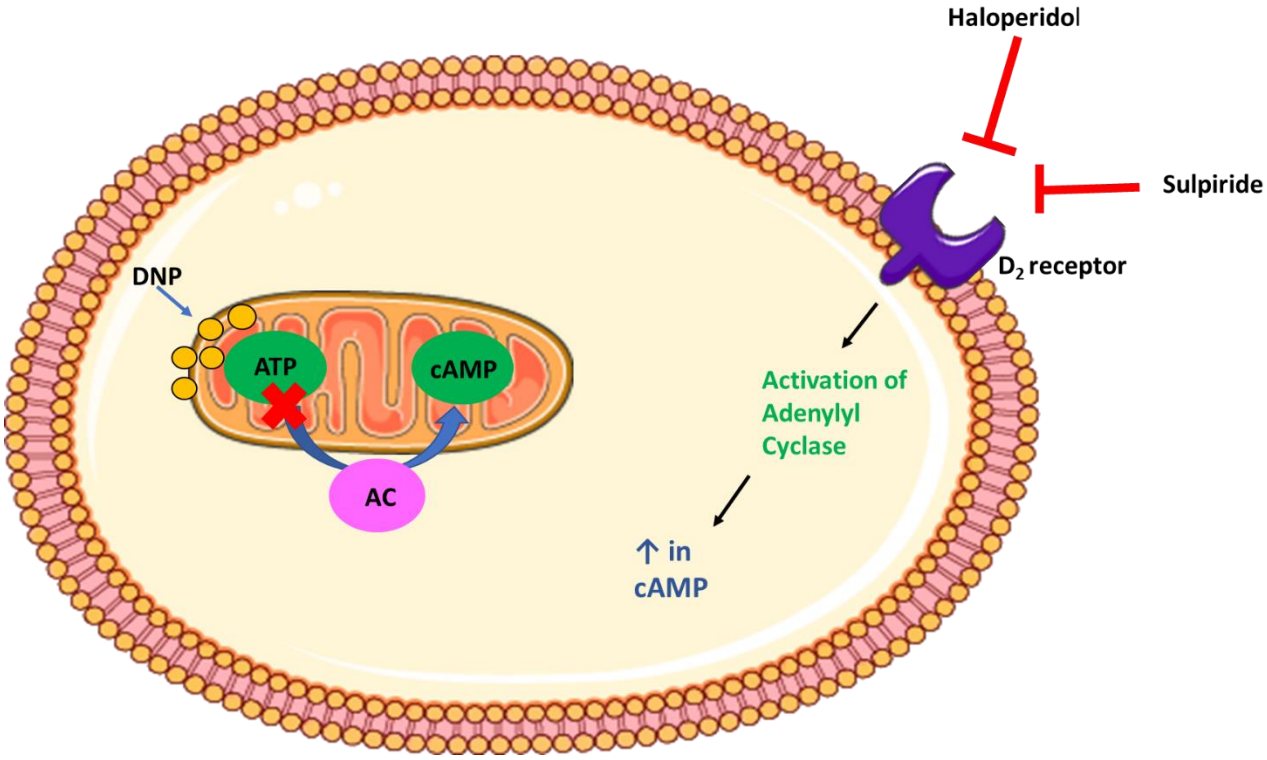

Figure 5.7. Schematic of the mechanism of action of DNP within the cell, and the potential mechanism by which haloperidol and sulpiride are exerting their effects to reverse toxicity.

6. Discussion

Being an endobenthic species, *L. variegatus* has been utilised in aquatic toxicity testing where they are exposed to sediment-associated compounds, and toxicity is determined by a change in behaviours (Ding et al., 2001; O'Gara et al., 2004; Vought & Wang, 2018). Throughout this project, we have demonstrated how *L. variegatus* and the methods used in the existing studies can be adapted for pharmacology purposes. With the maintenance and handling of *L. variegatus* being simplistic and their stereotypical behaviours and free locomotion quantifiable, we have optimised two assays both of which describe three behavioural endpoints; body reversal, helical swimming and free locomotion. These endpoints determine whether the compounds *L. variegatus* were exposed to have an effect. Although we cannot compare *L. variegatus* to a more complex *in vivo* model and we may be unable to replicate experiments carried out in models such as rodents, or even humans, in *L. variegatus*, there is a gap in the pharmacology field for an invertebrate model of this type. By utilising *L. variegatus* in pharmacology and drug development, we are following the principles to reduce, replace and refine the number of animals used in research, especially smaller invertebrate models such as *C. elegans*. As widely used as *C. elegans* are in biology and genetic research, they measure on average only 1mm in length (Meneely et al., 2019), meaning more individual *C. elegans* are needed per experiment compared to *L. variegatus*, plus there is a need for light microscopy to observe their behaviours, whereas no specialist equipment is required to observe *L. variegatus* behaviours. Like *L. variegatus*, *C. elegans* movements and behaviours have been a promising parameter for testing the toxicity of different chemicals, metals and organic compounds (Mortuza et al., 2013). The existing studies, literature and genetic data surrounding *C. elegans* have been an excellent framework for developing *L. variegatus* as a novel *in vivo* model for pharmacology, however by utilising *L. variegatus* we could potentially reduce the number of *C. elegans* and other invertebrate models used in research.

6.1. Chapter 1

Both lidocaine and quinine demonstrated dose-dependent responses on both stereotypical behaviours and free locomotion (Figures 3.1. A-B and 3.2. A-B). Existing literature and studies show that voltage-gated sodium ion channels are present in *L. variegatus*, as they play a role in their regeneration process (Alkhathlan, 2015) and treatment with voltage-gated sodium ion channel blockers can alter regeneration (Richmond, 2020). As lidocaine deactivates voltage-gated sodium ion channels, it reduces the excitability of neurons (Yang et al., 2020). It is possible that this mechanism is why there was a decrease in response to tactile stimulation at concentrations ≥0.5 mM. Although there is no existing data confirming that *L. variegatus* possess potassium ion channels, it is suggested that potassium channels are found in all living organisms and are found in all types of cells (Kim & Nimigean, 2016). When exposed to quinine there was an increase in free locomotion at 0.01 mM then effects became inhibitory at higher concentrations (Figure 3.3. F). This may be a result of off-target toxicity at concentrations >0.1 mM, However, quinine is known to be a peripheral muscle relaxant *in vivo* (Gisselmann et al., 2018) and so may be exerting its effect in a similar way to other *in vivo* models. Quinine is also known to act on muscular and neuronal nAChRs (Ballestero et al., 2005; Fukudome et al., 1998; Sieb et al., 1996). A study by Ballestero et al (2005) reported the effects of quinine on α9α10-containing nAChRs, showing that quinine blocked the acetylcholine-evoked responses in α9α10-injected *Xenopus laevis* oocytes (Ballestero et al., 2005). As previous studies have used nicotine to apply paralytic effects to *L. variegatus*, there is suggestive evidence that they possess nAChRs (Lesiuk & Drewes, 1999, 2001), meaning there is a possibility quinine may be exerting its effects through nAChRs.

Unlike lidocaine and quinine, dantrolene did not display a straightforward dose-dependent response. Despite this, there was a decrease in body reversal at 50 µM. Calcium ions are a well-known trigger of *in vivo* muscle contraction (Szent-Györgyi, 1975), However, dantrolene is known to inhibit calcium release in the skeletal muscle (Chamberlain et al., 1984). The response seen in *L. variegatus* body reversal when exposed to 50 µM of dantrolene may be due to a decrease of calcium ions when exposed to higher concentrations. If this process is similar to that observed in humans, dantrolene could be working as an antagonist, inhibiting the release of calcium ions and resulting in a decrease in the ability to respond to stimuli or perform behaviours. However, with an increase in free locomotion seen at 5 µM but no response observed at higher concentrations (Figure 3.3. F), it is possible that *L. variegatus* do not possess dantrolene binding sites within ryanodine receptors or their homologs. Again, with little information available surrounding *L. variegatus* pharmacokinetics and

pharmacodynamics these responses may differ depending on the absorption, distribution, metabolism, and excretion processes of each exogenous compound.

6.2. Chapter 2

There is strong evidence suggesting that *C. elegans* possess acetylcholine, dopamine, tyramine, octopamine, GABA and glutamate (Loer & Rand, 2010). Lybrand et al (2019) demonstrated that *L. variegatus'* escape circuit is a network of sensory interneurons electrically coupled to the central medial giant fibre, which is the interneuron used to perform body reversal (Lybrand et al., 2019). Electrical stimulation of the body wall initiated fast, short-lived spikelets in the medial giant fibre, which is suggested to be the result of giant fibre activation coupled to medial giant fibre dendrites. These contact sites have immunoreactivity with a glutamate receptor antibody, and the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dion diminishes MGF responses, meaning that *L. variegatus* possess glutamatergic pathways (Lybrand et al., 2019). As *L. variegatus* have been shown to possess glutamatergic pathways, this was a good basis to begin looking into the neuronal system in *L. variegatus*, to see whether they possess any neurotransmitter homologs.

As the amino acid neurotransmitter glutamate is excitatory (Zhou & Danbolt, 2014), we investigated dopamine. When exposed to increasing concentrations of dopamine we observed a dose-dependent response in *L. variegatus* body reversal and helical swimming and free locomotion (Figures 4.1. A-B & 4.1. F-G). Although little is known about the effects of dopamine on *L. variegatus* behaviours and movement, previous research by Puhl & Mesce (2008) determines that dopamine can activate the motor programme for crawling in the medicinal leech (Puhl & Mesce, 2008). Although our results show a progressive decrease in *L. variegatus* movement when exposed to increasing concentrations of dopamine, this is likely due to the difference in neural pathways between the two species. Alongside this, there is the possibility that if *L. variegatus* endogenously produce dopamine, exposing them to exogenous concentrations may affect their usual behaviour and movement, as demonstrated in our results. A previous study by Crisp et al (2010) looked at the role of biogenic amines, such as dopamine, in regulating pulsation of *L. variegatus* dorsal blood vessel (Crisp et al., 2010), this showed that when exposed to exogenous dopamine there was a 10% increase in *L. variegatus* pulse rate compared to resting pulse rate. These results are not unlike what is seen when exogenous dopamine is administered to human patients. As dopamine is vasostimulant, it can be used for the treatment of low blood pressure, low heart rate and cardiac arrest, as higher doses cause vasoconstriction and increased blood pressure (Sonne et al., 2022).

As dopamine influenced *L. variegatus* stereotypical behaviours and free locomotion we sought to determine whether they possess dopamine receptors homologs by examining the effects of dopamine receptor antagonists on behaviour. To do this we exposed *L. variegatus* to the non-selective dopamine D_1/D_2 receptor antagonist haloperidol (Kumari et al., 1999). When exposed to haloperidol a concentration-dependent response was observed as there was a decrease in body reversal and helical swimming at 50 and 100 µM (Figure 4.2. A-B). However, there was an increase in free locomotion at 10 µM before a gradual decrease was seen in the higher concentrations (25-100 µM) (Figure 4.2. F-G). This increase may be *L. variegatus* presenting with akathisia, a common side effect of haloperidol which is characterised by restlessness (Rahman & Marwaha, 2022). The inhibition seen in *L. variegatus* stereotypical behaviours and free locomotion may be haloperidol exerting its effects through dopamine-like homologs, as higher concentrations of haloperidol are often used as a rapid tranquiliser in psychosis-induced aggression or agitation (Ostinelli et al., 2017).

To further elucidate the impact of dopaminergic signalling in this organism, we examined the effects of the selective dopamine D_2 receptor antagonist, sulpiride (Memo et al., 1981) at equimolar concentrations to haloperidol. The results we obtained when exposing *L. variegatus* to sulpiride were dissimilar from those seen when exposed to haloperidol. Sulpiride had no effect on *L. variegatus* stereotypical behaviours or free locomotion. These results suggest that *L. variegatus* do not possess D_2 receptors, or that sulpiride may be unable to diffuse through their body wall. In humans, sulpiride is known to interact specifically with the dopamine D_2 receptor (Memo et al., 1981), whereas haloperidol is non-selective and can act on both dopamine D_1/D_2 receptors (Kumari et al., 1999). As exposure to haloperidol showed a dose-dependent behavioural response, but sulpiride had no effect on *L. variegatus* movements or free locomotion, it is likely that sulpiride may be too specific for this organism, as they may not possess dopamine D_2 receptor homologs.

Like mammals, invertebrates have different subtypes of dopamine receptors (Guo et al., 2015). Specifically in insects, four in total have been identified: D_1-like dopamine receptor (Dop1), invertebrate type dopamine receptors (INDRs or Dop2), D_2-like receptors (Dop3) and DopECR (Mustard et al., 2005; Srivastava et al., 2005; Watanabe et al., 2013). These receptors show differences in structure and function in different animals (Mustard et al., 2005; Romanelli et al., 2010), with one difference being that D_1-like dopamine receptors, INDRs and DopECR up-regulate intracellular cAMP levels, and D_2-like receptors down-regulate intracellular cAMP levels (Beggs et al., 2005; Mustard et al., 2005; Srivastava et al., 2005; Verlinden et al., 2015). *L. varigatus* lack of response to sulpiride could be due to a lack of dopamine-like receptor homologs, or specifically D_2-like receptors.

In *C. elegans* different classifications of GABAergic neurons are necessary for different behaviours (Mclntlre et al., 1993). When exposed to increasing concentrations of GABA we observed a dose-dependent response in *L. variegatus* body reversal and helical swimming movements (Figure 4.4. A-B). However, GABA did not have the same effect on *L. variegatus* free locomotion as there was no significant decrease observed as the concentrations increased (Figure 4.4. F). If *L. variegatus* possess similar GABAergic homologs to *C. elegans* this could be the reason for the difference in responses seen in the stereotypical behaviours and free locomotion. It is also possible that GABA is exerting its inhibitory effects through the same mechanism as in mammals. It was first demonstrated by David Curtis and Jeffrey Watkins that GABA inhibits the capability of mammalian neurons to fire action potentials (Purves et al., 2001). However, if this was the case, we would likely have observed changes in *L. variegatus* free locomotion unless GABAergic transmission within *L. variegatus* is confined to the medial fibres that play a role in body reversal and helical swimming. As previously mentioned, there is evidence suggesting that *L. variegatus* possess nAChRs (Lesiuk & Drewes, 1999, 2001). In *C. elegans* cholinergic motor neurons are known to excite body wall muscles in order to initiate body bends, however, they also excite GABAergic neurons that synapse onto the opposite body wall muscles. When acetylcholine excites and contracts a set of muscles, GABA is released to inhibit and relax the opposing muscles (White et al., 1976), meaning GABA elicits muscle relaxation (Schuske et al., 2004). If GABA plays a similar role in muscle relaxation in *L. variegatus* as it does in *C. elegans* this could explain the reason for the dose-dependent response we observed. Also, ivermectin irreversibly inhibits macroscopic and single-channel GABA-activated currents in *C. elegans* (Hernando & Bouzat, 2014) and a study by Hernando & Bouzat (2014) shows that ivermectin inhibits *C. elegans* muscle GABA and L-AChR receptors. Research carried out by Ding et al (2001) indicates that locomotor behaviours controlled by non-giant locomotor pathways, such as free locomotion, swimming and crawling (Ding, 2000), are more sensitive to ivermectin than behaviours controlled by giant locomotor pathways (Ding et al., 2001), such as body reversal and helical swimming (Ding, 2000). Having evaluated the results from previous studies by Ding et al (2001) and Hernando & Bouzat (2014), alongside our results, it is possible that GABAergic transmission in *L. variegatus* is confined to giant interneuron pathways.

Although there is no published evidence suggesting that *L. variegatus* possess GABAergic receptors, the receptors for GABA are well defined electrophysiologically in a range of other invertebrate species (Lunt, 1991). We furthered the investigation into whether *L. variegatus* possess neuroreceptors by exposing them to increasing concentrations of $GABA_A$ receptor antagonist bicuculline. Although invertebrate GABA receptors are less sensitive to bicuculline than $GABA_A$ vertebrate receptors (Lunt, 1991), when exposed to bicuculline we observed a dose-dependent response in *L. variegatus*

stereotypical behaviours (Figure 4.5 A-B). However, there was an increase in *L. variegatus* free locomotion when exposed to 5 µM of bicuculline, followed by a decrease in movement observed at the highest concentration of 250 µM (Figure 4.5 F). A range of literature and data suggests that invertebrate GABA receptors are not sensitive to bicuculline (Sattelle et al., 2003), for example, studies conducted on preparations of the housefly, heads of honey bees, identified cockroach, and locust neurones *in situ*, and unidentified insect neurones in culture all drew to the same conclusion, high concentrations of bicuculline fail to block GABA-gated Cl^- channels (Abalis et al., 1986; Beadle et al., 1989; Benson, 1988; Burrows & Laurent, 1993; Sattelle et al., 1988). This alongside there being little literature surrounding *L. variegatus* neuronal processes, brings us back to the question do *L. variegatus* possess GABA-like homologs? And raises the question is bicuculline exerting its effects via GABA-like receptors in *L. variegatus* or is there another mechanism of action? Further investigation into bicuculline and *L. variegatus* is needed on an *in vitro* level to support the behavioural response observed in our results.

To further elucidate evidence suggesting *L. variegatus* use neuronal transmission, we exposed them to increasing concentrations of inhibitory neurotransmitter glycine. Camien et al (1951) discovered high concentrations of glycine in the muscles of *Homarus vulgaris*, a type of European lobster, and *Maia squinado*, the European spider crab, suggesting that glycine, along with other amino acids, played a role in regulating osmotic pressure. Glycine is also found in a range of other invertebrates including several species of Arthropoda, Mollusca and Pelecypoda (Simpson et al., 1959). When we observed *L. variegatus* stereotypical behaviours and free locomotion in response to glycine the only change in body reversal and helical swimming, and free locomotion was observed at the highest concentration of 500 mM (Figures 4.6 A-B & 4.6 F). *L. variegatus* did not regain the ability to perform stereotypical behaviours or free locomotion after a 24-hour rescue period (Figures 4.6 C-D & 4.6 G), meaning the inhibition in movement seen was likely due to off-target toxicity. Glycine has been shown to significantly promote longevity in *C. elegans* (Liu et al., 2019) and a study by Miller et al (2019) shows that mice with elevated glycine levels had an increased life span. Liu et al (2019) determined that *C. elegans* exposed to 5 µM, 50 µM and 500 µM of dietary glycine showed an increase in median life span, however, when exposed to 5 mM and 10 mM of dietary glycine *C. elegans* life span was not prolonged. We exposed *L. variegatus* to thousand-fold the concentration that was needed to extend *C. elegans* life span and it did not have any effect on behaviours, with the exception of 500 mM. It is possible that glycine does not play a part in the regulation of *L. variegatus* stereotypical behaviours or movement, therefore moving forward, and to determine if *L. variegatus* possess glycine-like homologs, we would develop an assay to determine the life span of *L. variegatus* and whether dietary glycine would prolong the mean life span.

We then exposed *L. variegatus* ethanol, which is proposed to work as an indirect $GABA_A$ receptor agonist *in vivo* (Davies, 2003), binding to $GABA_A$ receptors and decreasing neuronal signalling (Davies, 2003). When exposed to increasing concentrations of ethanol there was a decrease in *L. variegatus'* ability to perform both stereotypical behaviours at concentrations ≥250 mM (Figure 4.7 A & B). However, after a 10-minute rescue period, *L. variegatus* recovered to baseline levels (Figure 4.7 C & D). Similarly, *L. variegatus* free locomotion was reduced when exposed to ethanol at concentrations ≥250 mM (Figure 4.7 F). Again, these results returned to baseline levels after a 10-minute rescue period (Figure 4.7 G). These results are similar to that seen in *C. elegans*, where a reversible effect was observed upon acute exposure to ethanol. The exogenous administration of ethanol resulted in dose-dependent changes in body bends responsible for *C. elegans* free locomotion as well as speed movement (Davies et al., 2003). A study by Mitchell et al (2007) shows that *C. elegans* thrashing behaviours are inhibited when exposed to concentrations of ethanol between 100 and 500 mM. Similar to these results, our data show that *L. variegatus* stereotypical behaviours and free locomotion was inhibited at concentrations of 250 and 500 mM. However, the inhibition in *C. elegans* thrashing behaviours reached a steady state after 5-minutes of exposure to ethanol (Mitchell et al., 2007), whereas we observed changes in *L. variegatus* movement after 10-minutes exposure to ethanol. *C. elegans* regained motility rapidly after the removal of ethanol, recovering to more than 70% of control within the first minute and regaining full motility after 2-minutes (Mitchell et al., 2007). Again, similar to this, our results show that *L. variegatus* recover completely regaining full ability to perform stereotypical behaviours and free locomotion 10-minutes after removal from ethanol. Throughout the behavioural and movement assays we used 10-minutes in drug-free artificial pond water as a rescue endpoint, as a result, we were unable to determine whether *L. variegatus* could recover from ethanol exposure before this endpoint. Despite this, we would consider this in future experiments to determine how quickly *L. variegatus* can recover. *Drosophila* also has a behavioural response when exposed to ethanol, as they exhibit an initial increase in locomotion, followed by incoordination, sedation and immobility (Wolf & Heberlein, 2003).

In humans, ethanol is known to act on a number of molecular targets in neurons and synapses throughout the brain and although ethanol does not have a specific mechanism of action, it has rapid acute effects on the function of the proteins involved in excitatory and inhibitory synaptic transmission (Abrahao et al., 2017). For example, it heightens the activity of cys-loop ligand-gated ion channels, such as $GABA_A$ and glycine receptors, but inhibits ionotropic glutamate receptors (Lovinger & Roberto, 2013). Previous studies have suggested that ethanol acts to increase GABA release from presynaptic terminals, resulting in enhanced synaptic inhibition (Siggins et al., 2005). To determine whether

ethanol exerts its effects via GABA-like receptors, moving forward we will *L. variegatus* to both ethanol and GABA in combination.

The effects of ethanol can be divided into two categories: acute and chronic. The CNS is mostly affected by acute exposure to ethanol as it results in depression of the CNS, causing sedation, impaired sensory and motor skills, impaired judgement, euphoria and uninhibited behaviour (Garg & Ketha, 2020). Acute functional tolerance (AFT) develops during short single exposure to ethanol and is not due to the changes in ethanol clearance (Wallace et al., 2007). We measured *L. variegatus* acute functional tolerance to ethanol. After being exposed to 500 mM of ethanol for 10-minutes, *L. variegatus* free locomotion was recorded. We then recorded the free locomotion at 20-minute intervals for 210 minutes and compared each time point to 10-minutes of ethanol exposure. Figure 4.8 shows that by the 210-minute time point there is a significant increase in movement compared with the initial 10-minute exposure. This response shows that *L. variegatus* are capable of developing tolerance to ethanol after acute exposure.

Moving forward with this project, we then developed a protein extraction and quantification assay, as well as a DNA extraction and quantification assay. These assays were mainly for proof of concept. We know to further our research and *L. variegatus* as an *in vivo* model, we will have to begin looking at their genetic makeup and evaluating the effects of drugs on a more molecular level, such as in *in vitro*. In a study by Vehniäinen & Kukkonen (2014) *L. variegatus* protein was extracted to determine whether the species possess ATP-binding cassette proteins. Having reviewed this paper, we developed a similar protein extraction assay, adjusting elements and making it suitable for our work. We exposed *L. variegatus* to ethanol before extracting protein as it is water soluble and readily available for use in the lab. We then performed SDS-PAGE then transferred the proteins from the gel onto a membrane. The gels were then stained with Coomassie blue and Ponceau (Figure 4.9). As this process was successful, it can be used in the future, as we further develop *L. variegatus* as an *in vivo* model on a more molecular level.

Following a paper by Gustafsson (2008) who had successfully extracted DNA from *L. variegatus*, we repeated this method and adapted it to our project. The worms were exposed to ethanol as it is completely water soluble and is readily available for use in the lab. DNA extraction was carried out on whole worms using E.N.Z.A.® Tissue DNA Kit (Omega Bio-Tek) per manufacturer's instructions, with one exception: elution buffer was heated to 55^{o}C rather than 70^{o}C. We then went on to quantify the DNA samples using a Nanodrop spectrophotometer (Table 4.1). Again, as this process was successful, we have a protocol in place for beginning to look at more than just the behavioural effects of drugs and compounds.

6.3. Chapter 3

Next, we sought to determine the toxic effects exerted by popular weight loss drug 2,4-dinitrophenol. By exposing *L. variegatus* to DNP alone and monitoring the behavioural effects, then exposing *L. variegatus* to DNP concurrently with a potential antidote therapy we were able to determine whether the worms could recover from DNP poisoning. This would also give us some insight into what sort of molecular and cellular processes *L. variegatus* possess, and whether the specific antidote therapies we chose for this study are exerting their effects through chemical or receptor-mediated interactions.

There has been an evident increase in deaths related to DNP toxicity in the UK, and it is driven by illegal distribution via internet sales (Holborow et al., 2016). DNP has been circulated throughout Wales and England, causing 23 deaths between 2012 and 2018 (Thomas, 2018). A Public Health Link by the Chief Medical Officer for Wales stated that the number of DNP poisoning cases and documented fatalities have significantly increased since 2012 (Atherton, 2019). Up until the end of March 2019, there had been 120 cases of systemic DNP exposure, with 98% of these cases occurring since 2012 (Atherton, 2019).

When exposed to increasing concentrations of DNP there was a dose-dependent decrease observed at concentrations ≥5 µM (Figure 5.1. A-B & F). After a 24-hour rescue, both stereotypical behaviours and free locomotion were inhibited at 25 and 50 µM (Figure 5.1. C-D & G), which is likely a result of toxicity. These results are similar to those of Barnes, 1955, who used isolated rat diaphragms in a study to determine the effect of DNP on muscle contractions and response to stimuli. Barnes's findings show that the muscle contractions are followed by slow muscle relaxation, resulting in the inability to respond to a stimulus (Barnes et al., 1955). Similarities were shown in *L. variegatus* that were exposed to 25 and 50 µM of DNP as they did not fully recover. This indicates that DNP has prolonged toxic effects on *L. variegatus* stereotypical behaviours and free locomotion at higher concentrations. Like humans, almost all eukaryotes have mitochondrion, which the main function is to supply energy in the form of ATP (Roger et al., 2017). Although there is no evidence suggesting that the mitochondria found in the cells of *L. variegatus* use the same process as that found in humans, DNP may use the same mechanism of action, uncoupling mitochondrial phosphorylation (Petróczi et al., 2015).

Currently, there is no bedside antidote for DNP poisoning, and all management strategies are based on case reports and medical expert opinions (Grundlingh et al., 2011b). Case studies show that dantrolene has been successful in reducing intracellular Ca^{2+} levels, and limiting heat production. Cooling with ice packs and cold intravenous fluids are also used when trying to treat DNP toxicity, as

well as the use of benzodiazepines to treat agitation and possible seizures (Kopec et al., 2019b). The National Poisons Information Service (NPIS) also recommend dantrolene for DNP toxicity, especially where there is muscular hyperactivity (Toxbase, 2020).

Based on the results obtained by previously exposing *L. variegatus* to dantrolene, a treatment used for malignant hyperthermia (Krause et al., 2004), and in the treatment of DNP poisoning (Kopec et al., 2019b), we exposed *L. variegatus* to DNP followed by dantrolene. Much like when being exposed to DNP alone, there was a dose-dependent decrease in *L. variegatus* stereotypical behaviours at ≥5 µM after 10-minutes of exposure to DNP and dantrolene in combination (Figure 5.2 A-B). After a 24-hour rescue, a decrease in stereotypical behaviours is still seen at 50 µM (Figure 5.2 C-D), which is likely due to toxicity. These results show that dantrolene is capable of reversing the effects of DNP at concentrations ≤25 µM. A significant decrease in free locomotion is seen only at 50 µM (Figure 5.2 F), whereas when exposed to DNP alone a decrease in movement is seen at ≥5 µM. After a 24-hour rescue period, *L. variegatus* free locomotion returns to baseline levels at all concentrations (Figure 5.2 G). This is promising as the results show that dantrolene inhibits the effects of DNP on *L. variegatus* free locomotion. After 24-hours *L. variegatus* response to stimuli returned to baseline levels at 25 µM. Despite these results, the use of dantrolene for DNP treatment is controversial. Although dantrolene is an established treatment for malignant hyperthermia, the molecular mechanism of hyperthermia in malignant hyperthermia and DNP toxicity is different (Bartlett et al., 2010). This being said, malignant hyperthermia is the uncontrollable release of calcium from skeletal muscle sarcoplasmic reticulum, which leads to continuous muscle contraction. This muscle contraction generates a depletion of ATP, increasing oxygen consumption, carbon dioxide production and heat (Watt & McAllister, 2022). This is similar to one mechanism of heat production seen in DNP toxicity, which is the inhibition of ATP production due to uncoupling of oxidative phosphorylation, leading to a decrease in calcium transport which results in the build-up of intracellular calcium, muscle contractions and heat production (Kopec et al., 2019). It is possible that dantrolene is exerting its effects through the same mechanism as in mammals. However, based on the findings of Plater and Harrison, 2019 who suggested basic drugs, such as haloperidol and quinine, can form an acid-base complex with DNP (Plater & Harrison, 2019), dantrolene may be also reversing the effects of DNP based on this theory. Dantrolene is a basic drug with a pKa of around 7.5 (National Center for Biotechnology Information, 2022), meaning it could potentially be forming an acid-base complex with DNP, however, it is a weaker base than quinine and haloperidol, which is why it may not be able to reverse the toxic effects of DNP at the highest concentration of 50 µM.

Plater and Harrison (2019) demonstrated that quinine and haloperidol are capable of transforming the chemical structure of DNP on the basis that they will form an acid-base complex. As DNP has a pKa

of 4.0, it is considered a very acidic drug, being around 8-10 times more acidic than acetic acid (Plater & Harrison, 2019). With quinine having a pKa of 8.55 (National Center for Biotechnology Information, 2022), and haloperidol having a pKa of 8.66 (El Tayar et al., 1985), this makes them very basic compounds meaning they have the potential to form an acid-base complex with DNP, reducing the availability of DNP in the system and relieving the symptoms of toxicity (Plater & Harrison, 2019).

As we had previously exposed *L. variegatus* to quinine, we determined the NOAEL as 0.1 mM. Following on from the work of Plater & Harrison (2019), we exposed *L. variegatus* to increasing concentrations of DNP followed by 0.1 mM of quinine.

When exposed to DNP and quinine in combination there was no significant difference compared to baseline levels, however after 10-minutes in drug-free artificial pond water, there was a change in *L. variegatus* ability to perform body reversal at concentrations ≥25 mM (Figure 5.3 C), and helical swimming at concentrations ≥5 mM (Figure 5.3 D). These results then returned to baseline levels after 24-hours (Figure 5.3 C-D). Unlike the stereotypical behaviour assay, a decrease in *L. variegatus* free locomotion was seen after exposure to DNP and quinine in combination at concentrations ≥25 mM (Figure 5.3 F). *L. variegatus* recovered fully after a 24-hour rescue period (Figure 5.3 G). These results confirm Plater & Harrison's hypothesis that quinine is capable of adjusting the chemical structure of DNP, forming an acid-base complex, and reversing the effects of DNP toxicity.

Following on from this, and having previously exposed *L. variegatus* to increasing concentrations of haloperidol, we determined the LOAEL of haloperidol for increasing movement as 10 µM and the LOAEL for inhibiting movement as 25 µM. When exposed to increasing concentrations of DNP followed by 10 µM haloperidol, there was a significant decrease in *L. variegatus* stereotypical behaviours at ≥12.5 µM. These results persisted after 10-minutes but returned to baseline levels after 24-hours in drug-free artificial pond water. This differed from when *L. variegatus* were exposed to DNP followed by 25 µM, as a decrease in response to stimuli was seen at ≥5 µM, this is likely due to the concentration of haloperidol being higher as it may have side effects. However, after 10-minutes in drug-free artificial pond water, the decrease in response seen at 5 µM had returned to baseline levels, whereas a decrease remained at concentrations ≥12.5 µM. After 24-hours in drug-free artificial pond water, results returned to baseline levels. Much like the stereotypical behaviour assay, *L. variegatus* were affected at lower concentrations when exposed to DNP followed by 25 µM of haloperidol, compared to DNP followed by 10 µM of haloperidol. When exposed to DNP followed by 10 µM of haloperidol, a decrease in *L. variegatus* movement was seen at ≥25 µM. Again these, results persisted after 10-minutes in drug-free artificial pond water but returned to baseline levels after 24-hours. Whereas, when exposed to DNP followed by 25 µM of haloperidol, the decrease in *L. variegatus* movement was

seen at ≥12.5 µM. However, after 24-hours *L. variegatus* free locomotion had returned to baseline levels.

Not only do these results confirm the work of Plater and Harrison (2019), but they support the work of Gatz & Jones (1970), who set out to determine whether haloperidol provided *in vivo* protection against hyperpyrexia and lethal effects of DNP using adult male Sprague-Dawley rats (Gatz & Jones, 1970). They determined that haloperidol did provide *in vivo* protection against the effects of DNP when administered 0.35 mg/kg 6 hours pre-exposure or 0.7 mg/kg 18 hours pre-exposure (Gatz & Jones, 1970). Gatz & Jones (1970) also discuss the possible mechanisms haloperidol uses to diminish the toxic effects of DNP. One potential mechanism is that haloperidol, being a potent neuroleptic, can decrease the permeability of several biological membranes to a variety of inorganic and organic molecules (Gatz & Jones, 1970; Seeman & Bialy, 1963). Alongside this, there is potential that haloperidol is transported to and accumulates in the mitochondria, decreasing the entrance of DNP into the mitochondria. Consequently, the penetration of DNP through the membrane could be diminished, inhibiting the toxic effects of DNP (Gatz & Jones, 1970). Also, a study by Roszell & Horita (1975) showed haloperidol to have slight hypothermic effects on rabbits with LSD-induced hyperthermia (Roszell & Horita, 1975). As hyperthermia is one of the most common symptoms of DNP toxicity, haloperidol could be using the same mechanism as seen in Roszell & Horita's study, however, this would need further study into *L. variegatus* temperature when administered DNP and haloperidol.

To determine whether there is a molecular explanation for haloperidol's mechanism in relieving the toxic effects of DNP, we exposed *L. variegatus* to DNP followed by selective dopamine D_2 receptor antagonist, sulpiride. This also added to our investigation into the presence of neurotransmission and neuroreceptors in *L. variegatus*. *L. variegatus* had previously been exposed to sulpiride, however, displayed no signs of toxicity or any significant changes compared with baseline levels. Although sulpiride did not have any effect on *L. variegatus* stereotypical behaviours or free locomotion, when exposed to DNP and sulpiride in combination, the results were similar to those seen when *L. variegatus* were exposed to DNP and haloperidol in combination. There was a significant change in behaviours at ≥5 µM (Figure 5.6 A-B) after exposure to DNP and sulpiride in combination, and these results continued after 10-minutes in drug-free artificial pond water. However, after a 24-hour rescue period, *L. variegatus* behaviours returned to baseline levels (Figure 5.6 C-D). There was a significant decrease in *L. variegatus* free locomotion at concentrations ≥25 µM (Figure 5.6 F), but again these returned to baseline levels after 24-hours in drug-free artificial pond water (Figure 5.6 G). Much like haloperidol, sulpiride provided *in vivo* protection against the toxic effects of DNP.

Brain catecholamines are considered to be involved in thermoregulation, with various studies having investigated the connection between dopamine and thermoregulation in the preoptic area and anterior hypothalamus (Hasegawa et al., 2005). A study by Nguyen et al (2019) shows that sulpiride significantly reduces methamphetamine-induced hyperthermia in mice (Nguyen et al., 2019). With hyperthermia being one of the major symptoms of DNP toxicity, sulpiride may use the same mechanism of action to reverse the effects of DNP as it does to reduce methamphetamine-induced hyperthermia. Having said this, sulpiride is also a basic drug, with a pKa of 9.12 (National Center for Biotechnology Information, 2022). This means that like both haloperidol and quinine, sulpiride is capable of forming an acid-base complex with DNP, neutralising it to salt and reducing the toxic effects.

7. Final summary

The use of *in vivo* models can contribute valuable information and insights into drug discovery and development, however, there is framework put in place to ensure more humane research and principles to replace, reduce and refine the number of animals used in research (Tannenbaum & Bennett, 2015).

For many years the use of animals in research has played a pivotal role in the progression of medical science and pharmacology. Although every year millions of animals are used for research purposes, many of the species used are tightly regulated by ASPA. Despite this, most invertebrate models are not covered by ASPA meaning they are under far less regulatory constraint and are often less expensive to culture and maintain. These can, therefore, provide valuable insight into *in vivo* mechanisms and remain an underutilised *in vivo* tool for pharmacology and toxicology research.

Throughout this project, we have utilised *L. variegatus* as a novel *in vivo* model for pharmacology testing. In doing so, we have optimised several assays, including both the stereotypical behaviour and free locomotion assay, acute functional tolerance assay, protein extraction and quantification, and DNA extraction and quantification. Although *L. variegatus* is a non-traditional model and genetic data is not readily accessible as other models, such as *C. elegans*, this species of aquatic worm has its advantages. *L. variegatus* is easily acquired from exotic fish food stores and laboratory-reared in aquariums containing artificial pond water, meaning they are inexpensive and easy to maintain. Alongside this there is no call for special husbandry as with rodents and other larger models, therefore *L. variegatus* can be cultured in most laboratories, including research and educational institutions.

This project documents the behavioural effects of diverse pharmacological compounds on *L. variegatus* response to stimuli and free locomotion. An advantage of using *L. variegatus* for both research and education is that they possess unique stereotypical behaviours that can be scored and easily quantified without the need for specialist equipment. The area covered by *L. variegatus* free locomotion can also be analysed using the free-to-use software ImageJ. Both are straightforward methods that can be used to observe the effects of drugs and compounds *in vivo*. By utilising these assays and reviewing the data obtained, we have determined that *L. variegatus* possess some sort of pharmacokinetic process.

Although most compounds administered throughout this project had an effect on *L. variegatus* response to stimuli or free locomotion, and both protein and DNA extraction were successful, there is still a need for further research to elucidate the full pharmacokinetic and pharmacodynamic profile of this novel *in vivo* model.

We recognise that the experiments we have conducted on *L. variegatus* throughout this project may not replicate the complexity of higher animals and experiments utilising invertebrates will not fully replace studies in vertebrate species, such as rodents. That being said, *L. variegatus* do have the potential to replace smaller invertebrate models where specialist equipment is needed to visualise them or to reduce the number of specimens needed per experiment. Although a fairly new model, we are not just limited to behavioural studies in *L. variegatus*, having successfully extracted and quantified both their protein and DNA, this opens up more avenues for us to proceed. More evidence, research and development are needed before *L. variegatus* become a well-known and utilised model within the pharmacology field, however, as we have demonstrated throughout this project, the potential is there.

8. Future directions

This project focused mainly on exogenously administered neurotransmitters and their antagonists and the behavioural effects these compounds have on *L. variegatus*. Moving forward we would administer these in combination. For example, haloperidol is a D_2 receptor antagonist. If we were to expose *L. variegatus* to increasing concentrations of dopamine, followed by the no observable adverse effect level of haloperidol this could give us a better insight into whether *L. variegatus* possess dopamine receptor homologs. This type of assay could also be repeated for dopamine and sulpiride, as well as other drug combinations.

L. variegatus are a species of annelid. Although there is no specific evidence suggesting *L. variegatus* as a species possesses serotoninergic receptor homologs, serotoninergic neurons have been localised in other annelids with the use of classical histochemical methods (Hessling et al., 1999). Alongside this, research carried out by Nentwig (2007) suggests that low concentrations of the selective serotonin reuptake inhibitor, fluoxetine, increase *L. variegatus* reproduction. Based on these findings, and to further our understanding and research into neurotransmission within *L. variegatus*, we would expose *L. variegatus* to serotonin and fluoxetine separately to see the behavioural response, then expose them to serotonin and fluoxetine in combination to see if there are any changes in response. Also, having reviewed Nentwig's (2007) paper, it would be an idea to develop a reproduction assay. In doing so, we would be furthering the knowledge of the effects of compounds on *L. variegatus* behaviours, as we would not be restricted to movement in response to stimuli and free locomotion.

L. variegatus have been one of the earliest annelids used in regeneration research (Acosta et al., 2021). Work by Bonnet (1745) shows that a single worm can be cut into 16 pieces, with each piece regenerating a completely new worm. In a review by Acosta et al (2021) the process of *L. variegatus'* ability to regenerate is discussed. The review explains the 5 stages of *L. variegatus* regeneration process: 1) wound healing, 2) Blastema formation, 3) Blastema differentiation, 4) Resegmentation, and 5) Growth (Acosta et al., 2021). This review gives us a brilliant insight into the cell processes used in regeneration and what kind of cells and tissues *L. variegatus* possess, showing that the structure of this organism is more complex than some may think. Moving forward, using the studies reviewed by Acosta (2021), we aim to optimise and develop a suitable regeneration assay. Worms cultured in the laboratory never reach sexual maturity or produce cocoons, therefore reproduction under laboratory conditions is always via asexual fragmentation. This is when an individual worm spontaneously splits into two or more fragments (Drewes, 2004). This means we would need to observe and determine the mean regeneration time for a group of selected worms. We would then expose *L. variegatus* to different drugs to see whether it would inhibit, decrease, or increase the regeneration time.

L. variegatus also possess a behaviour known as autotomy. Similar to the regeneration process, *L. variegatus* divide into fragments and then regenerate into whole worms, however with autotomy*, L. variegatus* rapidly divide into fragments as a result of threatening stimuli or direct damage (Lesiuk & Drewes, 1999). A study by Lesiuk & Drewes (1999) determined that this reflective and motor response that *L. variegatus* possess can be induced by adequate stimulation in the form of sudden body compression. However, Lesiuk & Drewes (1999) also determined that autotomy in *L. variegatus* is an all-or-nothing process. Near the point of compression, segments either quickly separated from one another or remained connected and fully intact (Lesiuk & Drewes, 1999). Using this work as a basis we aim to determine whether specific drugs would inhibit segmental autotomy in the response to sudden body compression, or whether certain concentrations of drug would induce segmental autotomy, without physical stimuli.

Our understanding of how ethanol regulates brain function and behaviour is limited as ethanol does not act through a specific receptor (Wolf & Heberlein, 2003). However, what we do know is that ethanol enhances GABAergic neurotransmission in vertebrates and GABA receptors are a major target of ethanol's actions (Sullivan et al., 2010). As we have previously exposed *L. variegatus* to GABA and ethanol, moving forward we would administer these both in combination to see whether there is a change in behavioural response. As ethanol potentiates GABAergic transmission, we would then expose *L. variegatus* to GABA, ethanol and $GABA_A$ receptor antagonist bicuculline, to see whether there is a difference in responses between GABA and ethanol in combination and GABA, ethanol and bicuculline in combination. By doing this we can see whether ethanol potentially enhances the effects of GABA in *L. variegatus* and whether bicuculline can stop those effects.

Having determined that *L. variegatus* can develop a tolerance to ethanol after acute exposure, we aim to develop a place preference assay, which would allow us to observe the chronic effects of ethanol on *L. variegatus*. A study by Lee et al (2009) shows that *C. elegans* develop a preference for ethanol after chronic exposure. We would look to repeat the assay used in this study, making adjustments to suit the aquatic *in vivo* model that is *L. variegatus*. *C. elegans* pre-exposed to ethanol for 4-hours developed a significant preference for ethanol (Lee et al., 2009), however as *L. variegatus* is an aquatic species and ethanol is water soluble, we would look to culture *L. variegatus* in ethanol.

Chronic drug exposure can lead to compulsive drug-seeking habits (Everitt & Robbins, 2005), if these drug-seeking habits were seen in *L. variegatus* when exposed to ethanol for a prolonged period this could allow us to move forward with our research into neurotransmission *in L. variegatus* and developing them as a novel *in vivo* model. As previously mentioned, we would aim to expose *L. variegatus* to GABA, ethanol and bicuculline to see if there are any behavioural changes. Other drugs

we aim to administer in combination to observe *L. variegatus* stereotypical behaviours and free locomotion are GABA and baclofen, ethanol and baclofen and GABA, ethanol and baclofen. Baclofen is a $GABA_B$ receptor agonist which has been used in managing alcohol addiction (Gorsane et al., 2012). In both rats and mice, baclofen has been reported to suppress ethanol-induced locomotor stimulation, binge-like and relapse-like drinking, alcohol seeking and operant oral alcohol self-administration, alongside this it has been reported that several of these effects are mediated by $GABA_B$ receptors in the ventral tegmental area (Colombo & Gessa, 2018). If we are successful in developing a place preference assay, we will observe *L. variegatus* preference when pre-treated with ethanol, then when pre-treated with ethanol followed by exposure to baclofen to see if there are any distinct differences and if *L. variegatus* do develop ethanol-preference, whether it can be reversed.

There is limited literature and studies surrounding *L. variegatus*, however, we have reviewed studies that suggest the presence of cholinergic transmission in *L. variegatus* and other invertebrates (Gerschenfeld, 1973; Lesiuk & Drewes, 1999; Sardo & Soares, 2010). Leisuk & Drewes (1999) demonstrated a dose-dependent decrease in *L. variegatus* autonomy in relation to increased concentrations of nicotine. Alongside this, the acetylcholine agonist Imidacloprid has been shown to decrease *L. variegatus* survival, inhibit behaviour, interfere with growth processes and shorten lifespan (Sardo & Soares, 2010). These studies, and with reason to believe quinine may be exerting its effects through nAChRs, exposing *L. variegatus* to acetylcholine and observing their stereotypical movements and free locomotion will further our research into the presence of neurotransmission within *L. variegatus* and further elucidate the species as an *in vivo* model. Moving forward with this idea, we would then expose *L. variegatus* to nicotine alone, and then acetylcholine and nicotine in combination, observing any differences in behaviours.

One of the limitations of this project was the lack of available genetic data. Although this has been an issue throughout the project, we have taken on a "starting from scratch" approach allows us to be less biased towards our data. Having successfully extracted protein from *L. variegatus*, we aim to detect specific protein molecules. To do this we will perform a western blot, which will identify specific proteins using antibodies. This technique utilises three elements to identify proteins, these being separation by size and transfer to a solid support, which we have been successful in doing previously, and probing using antibodies to target the specific protein. We also aim to use Polymerase Chain Reaction (PCR) to amplify DNA. If this was successful, we could then move on to electrophoresis which would separate the DNA molecules based on their size.

Again, with little existing data surrounding *L. variegatus* genetic make-up, it is not known whether the compounds we expose *L. variegatus* to are affecting their behaviour due to chemical or molecular

processes. One example of this is how haloperidol works to reverse the toxic effects of DNP. Although Plater & Harrison suggest that haloperidol forms an acid-base complex with DNP, there is a molecular theory. DNP works by uncoupling mitochondrial OXPHOS (Grundlingh et al., 2011), however, it is suggested that cAMP regulates OXPHOS in the mitochondria (Valsecchi et al., 2013). cAMP is generated by adenylyl cyclase (Zhang et al., 2016) and dopamine D_1-class receptors are known to initiate adenylyl cyclase activity leading to the production of cAMP (Beaulieu et al., 2015). With haloperidol being a non-selective dopamine receptor antagonist, on binding to the dopamine D_1 receptor, it may increase cAMP levels within the mitochondria, leading to the regulation of the DNP-mediated uncoupling of OXPHOS. Moving forward and to determine whether or not this theory is valid, we will develop an assay to measure the bioenergetics profile of *L. variegatus* at baseline, when exposed to DNP alone and when exposed to DNP and haloperidol in combination, allowing us to observe any changes in cAMP levels.

A study by Wang & Wang (2021) looked at the effect of bisphenol A on the pulse rate of *L. variegatus* dorsal blood vessel. Much like the behavioural assays we have developed during this project, the technique used to measure the dorsal blood vessel pulsation rate is easily quantifiable and is a commonly used endpoint for toxicity testing in both research and practical laboratory teaching (Lesiuk & Drewes, 1999). To further our knowledge of the effects of the compounds used throughout this project on *L. variegatus*, we aim to develop a pulse rate assay. This would give us useful insight into whether drugs have a similar effect on *L. variegatus* pulsation rate as they do on vertebrate models, including humans. For example, quinine which we have used in this project has been looked at as a potential anti-arrhythmic drug in humans, with several studies addressing the potential anti-arrhythmic effects in animal models (Sheldon et al., 1995). By optimising a pulse rate assay, we can measure the mean pulse rate of untreated worms, then expose them to increasing concentrations of quinine and compare the results to see if quinine has had any effects on *L. variegatus* pulse rate. This assay would be suitable for all drug compounds, especially those that are known to affect the cardiac system.

Moving forward with developing *L. variegatus* as an *in vivo* model we aim to further our knowledge of the organism on an in vitro level. In doing so we will develop an assay that will provide us with an insight into cell toxicity when exposed to certain compounds. Adenylate kinase is a small enzyme present in all eukaryotes, which plays a vital role in ATP regulation and is released upon cell death (Ionescu, 2019). Exposing *L. variegatus* to a toxic substance or drugs at cytotoxic concentrations would result in cytolysis and an increase in adenylate kinase. This increase can then be measured using a bioluminescent non-destructive cytolysis assay kit, such as ToxiLight™ BioAssay Kit. As the damaged cells release more adenylate kinase The ToxiLight™ reagent will display a higher light intensity, meaning a comparison can be made between pre-exposure and post-exposure. This assay would be a great expansion of the work we have previously done with toxic compound DNP, as well as furthering our insight into *L. variegatus* cell processes and allowing us to further validate this organism as an *in vivo* model for pharmacological and toxicological studies. Furthermore, by continuing the development of in vitro studies in *L. variegatus* will allow us to move towards whole-genome sequencing, solidifying the use of these invertebrate models across the pharmacology, medical and biosciences field.

Appendix

Appendix 1.1. Scoring sheet used to measure *L. variegatus* stereotypical behaviours.

Worm	Treatment	Body Reversal Movements					Helical Swimming Movements				
		1	2	3	4	5	1	2	3	4	5
A1	Baseline										
	Control										
	Rescue										
	24 Hour Rescue										
A2	Baseline										
	Rescue										
	24 Hour Rescue										
A3	Baseline										
	Rescue										
	24 Hour Rescue										
B1	Baseline										
	Rescue										
	24 Hour Rescue										
B2	Baseline										
	Rescue										
	24 Hour Rescue										
B3	Baseline										
	Rescue										
	24 Hour Rescue										

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