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Lumbriculus variegatus: Investigating a Novel *in vivo* Model for Substances of Abuse

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MSc In Medical and Health Care Studies by Research

Declaration and statements

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

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From the river to the sea, Palestine will be free.

Abstract

To replace, reduce and refine using vertebrates in pharmacological research, invertebrate *in vivo* models are required to continue investigating pharmacology in whole animal systems. This study presents the freshwater oligochaete worm *Lumbriculus variegatus* as a novel *in vivo* invertebrate model to study the behavioural and physiological effects of ethanol and nicotine, two widely used substances of abuse.

L. variegatus were administered ethanol, GABA, bicuculline, baclofen, nicotine, mecamylamine and tubocurarine. Behavioural responses were observed using stereotypical movement assays, measuring responses to tactile stimulation and free locomotion assays, measuring drug effects on unstimulated movement. Optimisation of the *in vitro* techniques Western blotting, gas-chromatography and acetylcholine assays measured heat shock protein (Hsp) and fatty acid expression as well as cholinergic activity, respectively.

Exposure to ≥250 mM ethanol significantly reduces *L. variegatus* free locomotion at 2 minutes. At 500 mM ethanol, worms demonstrate increased Hsp70 expression and develop acute tolerance. Chronic exposure to 100 mM ethanol increases worm body size and induces oleic acid expression. Pre-treatment with $GABA_A$ antagonist bicuculline (2.5 mM) reverses 100 mM ethanol-induced reduction in *L. variegatus* unstimulated movement. Pre-treatment with $GABA_B$ agonist baclofen (20 mM) reverses 100 mM ethanol-induced reduction in body reversal. *L. variegatus* express endogenous acetylcholine and acetylcholinesterase. Tubocurarine, a nicotinic receptor antagonist, reduces worm stimulated movement at ≥25 mM. Pre-treatment with receptor antagonist mecamylamine (100 μM) reverses nicotine-induced reduction in *L. variegatus* unstimulated movement. Pre-treatment with tubocurarine (10 μM) potentiates nicotine-induced reduction in unstimulated movement.

Whilst unable to fully replace the complexity of drug responses in vertebrate models, *L. variegatus* are able to establish drug dose response relationships, contributing to reducing and refining vertebrate models in pharmacological research. *L. variegatus* are advantageous over other invertebrate models of drug abuse such as *Caenorhabditis elegans* due to their larger size and the ability to culture *L. variegatus* in the laboratory.

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Presentations and Publications

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- The Effects of Acute and Chronic Ethanol Exposure in the Novel *in vivo* Research Organism, *Lumbriculus variegatus*

  Romessa Mahmood, Elis Roome, Kwang Lee, Aidan Seeley

  Presented at BPS Pharmacology 2022

  13 September 2022

Abbreviations

ACh Acetylcholine

AChE Acetylcholinesterase

AChR Acetylcholine receptors

ADME Administration, distribution, metabolism and excretion

ADH Alcohol dehydrogenase

AFT Acute functional tolerance

ALDH Aldehyde dehydrogenase

AMPK AMP-activated protein kinase

AP Action potential

ASPA Animals (Scientific Procedures) Act 1986

ATP Adenosine triphosphate

AUD Alcohol use disorders

BAC Blood alcohol concentration

BPA Bisphenol A

BSA Bovine serum albumin

cAMP Cyclic adenosine monophosphate

CNS Central nervous system

COSHH Control of substances hazardous to health

CPA Conditioned place aversion

CPP Conditioned place preference

Cyt*c* Cytochrome C

DALYS Disability adjusting life years

DNA Deoxyribonucleic acid

DBV Dorsal blood vessel

DMSO Dimethyl sulfoxide

DSM Diagnostic and Statistical Manual

ECL Enhanced luminol-based chemiluminescent

ETC Electron transport chain

GABA Gama-aminobutyric acid

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GIRK G-protein activated inwardly rectifying K^+

GPCRs G-protein coupled receptors

GSH Glutathione

HSF Heat shock factor

Hsp Heat shock protein

LD_{50} Lethal dose

LDH Lactate dehydrogenase

LGF Lateral giant fibre

LGIC Ligand-gated ion channel

LOAEL Lowest observable adverse effect level

mAChR Muscarinic acetylcholine receptors

MCR Mitochondrial respiratory chain

MEOS Microsomal ethanol oxidising system

MGF Medial giant fibre

NAc Nucleus accumbens

nAChR Nicotinic acetylcholine receptors

NCBI National Centre for Biotechnology Information

NMBA Neuromuscular blocking agent

NOAEL No observable adverse effect level

PCR Polymerase Chain Reaction

PIC Protease inhibitor cocktail

PNS Peripheral nervous system

PVDF Polyvinylidene difluoride

RDL Resistance to Dieldrin

RIPA Radio immunoprecipitation assay

ROS Reactive oxygen species

SA Self-administration

SDS-PAGE Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SEM Standard error of the mean

VBV Ventral blood vessel

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1. Introduction

Pharmacology is the study of how drugs, including medicinal and recreational, work and interact with the body and includes drug discovery and development to ensure their safe and effective use from an individual to a global scale (Currie, 2018).

Drug discovery and development starts when a gap within therapeutics for a certain disease or ailment has been identified (Hughes et al., 2011). By understanding the pathophysiology of the target disease, target molecules can be identified, optimised, tested, and licensed (figure 1.1.). This process can take up to 15 years (Hughes et al., 2011) and is currently estimated to cost around $2.6 billion per new drug target (Kiriiri et al., 2020).

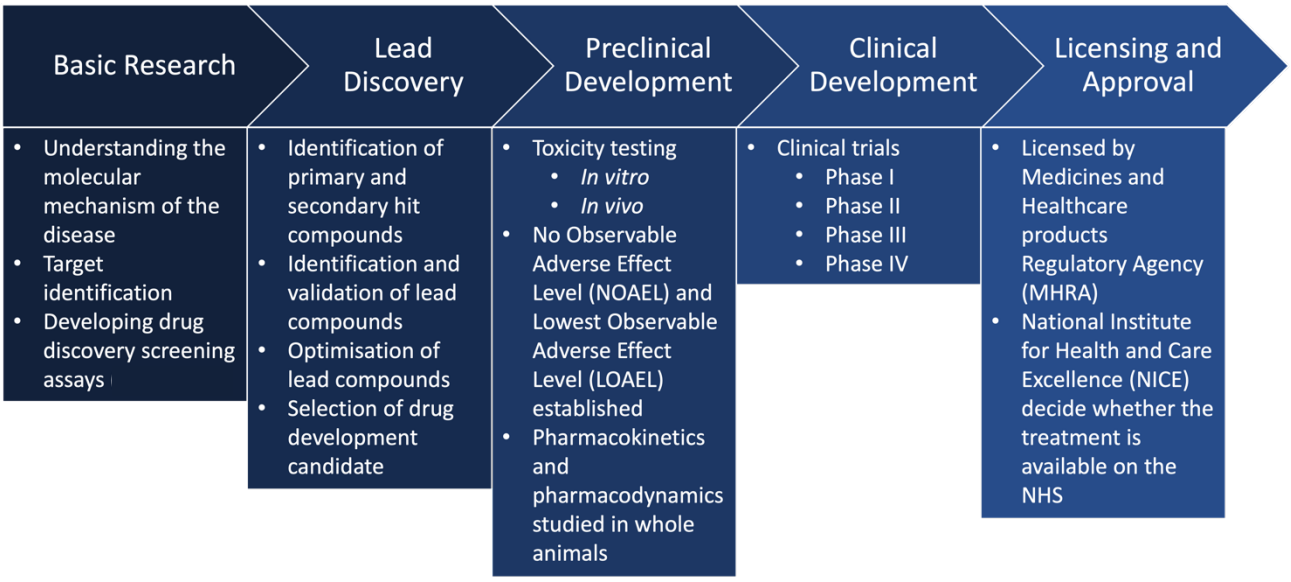


Figure 1.1. The drug discovery and development timeline. The journey from identifying a target to developing a licensed drug occurs in multiple stages and can take many years (adapted from Hughes et al. (2011); Kiriiri et al. (2020)).

When identifying the target at which the drug should act, drug compounds should be able to interact and induce a biological therapeutic response at this target. This response should be observable *in vitro*, Latin for "within the glass" and *in vivo*, Latin for "within a living organism" (Mattes, 2020). Once a target has been identified, identification of molecules with suitable pharmacological responses following interaction with the target occurs. Primary hit

compounds, molecules with which the desired pharmacological effects are observed, are modified to increase potency, and reduce adverse effects (Hughes et al., 2011). Through secondary assays, *in vitro* and *in vivo*, lead compounds are identified, optimised and a drug candidate is determined; this drug candidate will then move on through to preclinical studies. During preclinical studies, the drug absorption, metabolism, distribution, and excretion (ADME) are established (Hughes et al., 2011; Kiriiri et al., 2020). *In vitro* and *in vivo* toxicity tests are also conducted to ensure the safety of the drug on a human population to ensure that the drug can reach the next stage: Clinical Development. Only 1 in 10 drug compounds reach the clinical trials stage (Hughes et al., 2011). During the four phases of clinical trials, new drugs are administered to a human population, increasing in size at each phase, to establish dosing and observe any adverse effects (World Health Organisation, 2020).

1.1. *In vitro* and *in vivo* models in pharmacological research

In vitro experiments are procedures that are completed in non-whole organisms such as cells (Nikolic et al., 2018). Immortal cell lines are a popular *in vitro* model due to their ability to continuously undergo cell division without deterioration of the cells or disruption of the cell cycle (Maqsood et al., 2013). This makes them a cheaper, easier, and more consistent model compared to primary cell cultures which are made up of cells that are directly retrieved from an animal and therefore have a limited life span (Kaur & Dufour, 2012; Payne, 2023). There are a wide variety of cell lines available for use in research of the pathogenesis of many illnesses and disorders such breast cancer (Neve et al., 2006), osteoarthritis (Johnson et al., 2016) and Alzheimer's (Stoppelkamp et al., 2011). They can also be used to study the pharmacological profile, including the toxicity, of drugs ranging from anticancer drugs (Niu & Wang, 2015), opioids (McCarthy et al., 2001) to nicotine (Matsunaga et al., 2001) and cannabinoids (McCarthy et al., 2001). Cell lines, however, provide some disadvantages. They are unable to replicate how a drug may impact whole organism systems within a living organism and the risk of cross-contamination and genetic variation over an extended period of time result in heterogeneity meaning that later cells will not replicate the behaviour of the primary cells from which the line was created (Kaur & Dufour, 2012).

In vivo experiments are procedures completed using a living organism such as humans and animals and are normally carried out after *in vitro* studies (Dornell, 2022). In the drug discovery and development timeline, animal models play a vital role, especially in preclinical stages of pharmacological studies. *In vivo* studies observing drug pharmacokinetics, pharmacodynamics, and efficacy using animal models are necessary before proceeding on to trialling drugs in a clinical setting with a human population (Brake et al., 2017). Trialling drugs on an animal model also allows for the toxicity of the drug on whole organ systems to be observed. This will include a dose-escalation study in which models are assigned different doses of the drug and the efficacy or toxicity is observed (Le Tourneau et al., 2009). The results are graphed in dose-toxicity and dose-efficacy curves (Le Tourneau et al., 2009) from which the no observable adverse effect level (NOAEL), the highest drug concentration at which no pharmacological response is observed, and lowest observable adverse effect level (LOAEL), the lowest drug concentration at which a pharmacological response is observed, of a drug can be established (Zarn et al., 2011). Acute drug toxicity tests can also establish the median lethal dose (LD50) of a drug, where the dose administered will result in the death of 50% of the trial animals (Quiñones-Torrelo et al., 2001). These results can therefore ascertaining a safe but effective human starting dose during clinical trials (Polson & Fuji, 2012). Common *in vivo* models within pharmaceutical research include the *Rattu*s (Caroline Blanchard et al., 1988; Modlinska & Pisula, 2020), *Mu*s (Hankenson et al., 2011; West et al., 2000) and *Xenopus* (Ivorra et al., 2022; Villumsen et al., 2015; Whittemore et al., 1996) genera.

1.2. Replacement, Reduction and Refinement of *in vivo* models

Ethical challenges may be posed when using non-human living models such as animals. In 1960, Russell and Burch published the Three Rs Principles: Replacement, Reduction and Refinement (Figure 1.2). The premise of “Replacement” sets the expectation that where possible, in place of animal models, *in vitro* or *in silico* (computer system) models are used. Where the replacement of an animal model is not possible, “Reduction” ensures that there are fewer animal models used and that each model is used to its full potential to gather as much as data as possible (Russell & Burch, 1960). Where Replacement and Reduction have been implemented or are unable to be followed, “Refinement” ensures that any animal model used is bred and housed within suitable conditions (Russell & Burch, 1960). It also ensures

that any procedures carried out on the model will use the minimum number of animals and cause the least amount of pain or distress to those animals, for example using organisms with the lowest sentience but still achieving the same scientific output (Fenwick et al., 2009). Despite their proposal occurring during a time where the ethics of animal testing were not deemed as a priority, the Three Rs are implemented within ethical framework surrounding animal testing internationally today (Hubrecht & Carter, 2019).

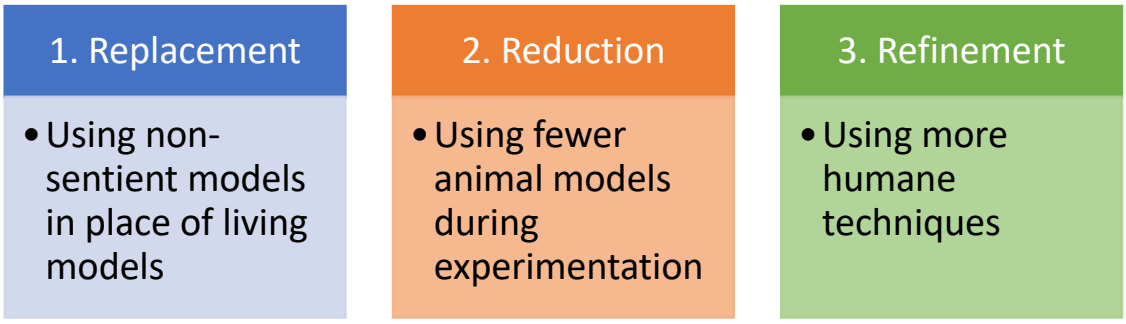


Figure 1.2. The Three Rs Principles. Each of the Rs should be implemented in order. Should replacing the animal models not be possible, fewer models should be used with more humane and refined techniques and procedures (figure adapted from Russell & Burch, (1960)).

Within the UK, in 1986, legislation was passed to protect the welfare of animal models within scientific research; the Animals (Scientific Procedures) Act 1986 (ASPA). ASPA protects both living non-human vertebrate and cephalopod models; a model can be classified as living until the brain has been permanently cut off from circulation or destroyed. It also includes embryos that are within their final third stage of gestation and fish and amphibian larvae once they are able to feed freely (Animals (Scientific Procedures) Act, 1986). Whilst cephalopod are invertebrates, their cognitive abilities have contributed to their use as animal models within neuroscience, cellular biology, and behavioural ecology research (Nakajima et al., 2018).

In 2021, there were a total of 3.06 million scientific procedures using animal models; this is a 26% decrease between 2015 and 2021 in Great Britain. Over 56% (1.72 million) of these procedures were classed as experimental procedures: the use of animals in scientific studies including basic research, treatment development and safety testing. Regulatory testing

includes assessment of safety and effectiveness of therapeutics and ensuring substances meet legal requirements and made up 21% of all experimental procedures (Figure 1.3. A) and 52% of regulatory testing was “toxicity and other safety testing” (Figure 1.3. B) (Home Office, 2022).

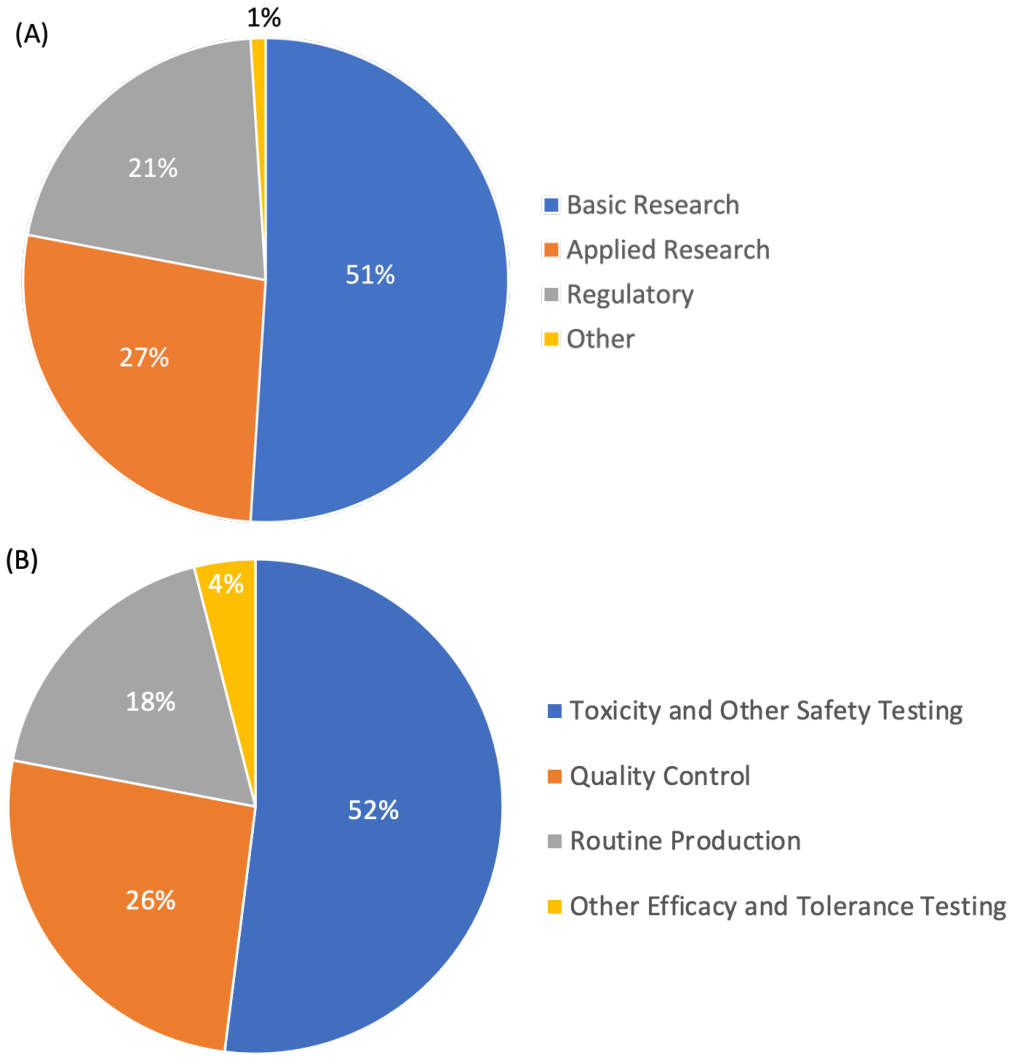


Figure 1.3. Purpose of experimental procedures in 2021. (A) A breakdown of the percentage of each experimental procedure by purpose. (B) A breakdown of the percentage of each regulatory procedure by sub-purpose (adapted from Home Office, 2022).

Within the UK, the most common animal models used for experimental procedures (including pharmaceutical testing) were mice (54%), fish (15%) and birds (14%) (Figure 1.4). Rats were the most used animal model for regulatory procedures with 97% being used in “toxicity and other safety testing” which includes pharmacological research (Home Office, 2022).

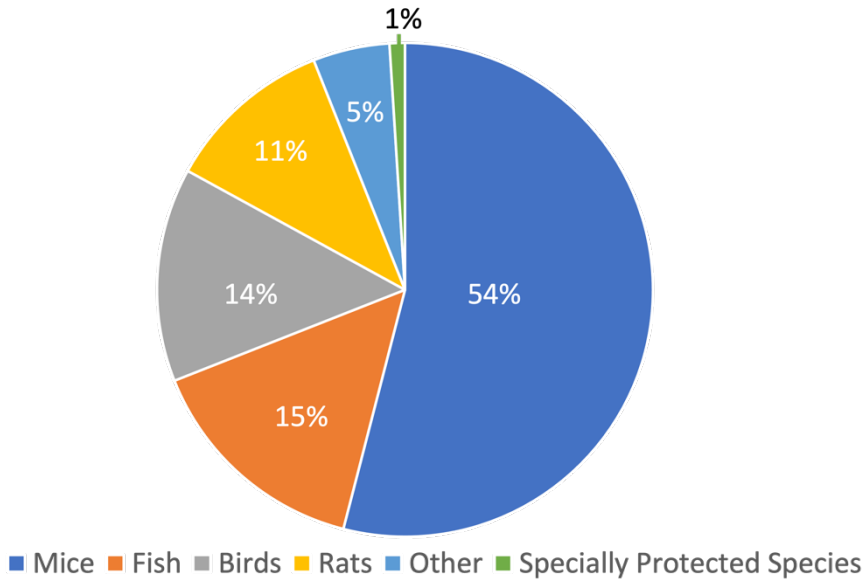


Figure 1.4. Species used during experimental procedures (2020). Specially Protected Species include Cats, Dogs, Horses, and Primates (adapted from Home Office, 2022).

1.3. Animal models in drug addiction

Drug abuse is the use of psychoactive compounds inappropriately, including consuming excess amounts, taking high doses and using the drugs in inappropriate settings, causing health or social problems (McLellan, 2017). Drug abuse can result in drug tolerance, dependence and addiction (Szalavitz et al., 2021). Tolerance is defined as experiencing a reduced effect of as drug when being repeatedly exposed to the same dose of the drug (Elvig et al., 2021). Dependence is defined as needing more exposure to a drug to avoid experiencing withdrawal symptoms when there is no access to the drug (Szalavitz et al., 2021). Drug addiction is defined as the continuous use of a drug despite any harmful consequences (McLellan, 2017). Drug tolerance will often lead to drug addiction due to the drug-taking individual needing to take more of the drug to feel the same effect (McLellan, 2017).

Drug addiction causes damage and disruption to the neurocircuitry within the human brain and is therefore classed a brain disease (Koob & Volkow, 2010). Whilst replicating the pathology of drug addiction in a nonhuman *in vivo* model comes with difficulties such as replicating environmental factors, genetics and behavioural predispositions to drug addiction, preclinical animal models are essential in understanding the pathophysiology of addiction so that more effective treatments may be developed (Kuhn et al., 2019). The ability to mimic

human behavioural responses following drug consumption is vital in a preclinical model when modelling addiction (Kuhn et al., 2019; Spanagel, 2017).

Despite the difficulties of replicating human genetics in animals, the genetic influence on the development of drug addiction has been widely explored using transgenic animal models. Transgenesis is the addition of foreign gene expression or removal of endogenous gene expression (Houdebine, 2007). In humans, dopamine receptors are involved in the development of drug addiction with inhibitory D_2-like receptor (D_2R) activity shown to be reduced following chronic ethanol (Volkow et al., 2017) and cocaine administration (Volkow et al., 2017), diminishing the dopamine response to the administration of psychostimulants. In both ethanol preferring and non-preferring rats, inducing an overexpression of D_2R by transferring the DRD2 gene decreased ethanol preference and ethanol drinking (Thanos et al., 2004). Inhibitory D_3-like receptor (D_3R) expression has been seen to increase in the human brain following cocaine (Prieto, 2017) and amphetamine administration (Boileau et al., 2017). In mice, ethanol administration also increases D_3R expression (Leggio et al., 2014) and when the DRD3 gene is overexpressed, this reduces both ethanol preference and voluntary ethanol intake (Bahi & Dreyer, 2014). These results support the hypothesis that dopamine receptors play an active role in the development of drug addiction and show that the genetic influences of dopamine receptors can be translated from humans to animal models.

Modelling drug addiction using animal models can include non-contingent models, including behavioural sensitisation and conditioned place preference (CPP), and contingent models, such as lever pressing or nose port entry. The difference between the two models is the mode of drug administration: whereas in non-contingent models, the drug is administered by the researcher during the study, in contingent models, the drug is self-administered (SA) during the study. Models that aim to replicate the motivation to take drugs and relapse and the role of other rewards, such as food, in drug-seeking behaviour are also commonly used (Kuhn et al., 2019).

1.4. Invertebrate models for ethanol and nicotine

Ethanol and nicotine are two common addictive drugs, which have been studied using both vertebrate and invertebrate models. Whilst drug addiction studies have commonly used vertebrate models (Spanagel, 2017), the use of invertebrate models including *Caenorhabditis elegans* (*C. elegans*, roundworm), *Drosophila melanogaster* (*D. melanogaster,* fruit flies), and *Apis mellifera* (honeybees) (Scholz & Mustard, 2011; Søvik & Barron, 2013) has also been explored.

CPP is a commonly used model that allows researchers to observe the rewarding or adverse effects of a drug by conditioning the animal to associate a subjective response to the drug to a specific place (Kuhn et al., 2019). First developed by Beach (1957) to demonstrate morphine addiction in rats by placing them in a Y-choice discrimination box, variations of CPP have been carried out with rodents to suggest the rewarding effects of other drugs of abuse, including cocaine (Ettenberg et al., 1999), amphetamine (Bardo et al., 2001), nicotine (Natarajan et al., 2011) and alcohol (Pati et al., 2019). CPP has also been used to establish the role of neurotransmitter pathways in drug rewarding systems, such as the GABAergic pathways in ethanol-induced CPP (Chester & Cunningham, 1999) and the cholinergic pathways in cocaine-induced CPP (Shinohara et al., 2014). This in turn has allowed researchers to identify which receptor systems to target when developing therapies for drug addiction. CPP is a beneficial model when considering its use for invertebrate species as it has since been adapted in studies using both *C. elegans* and *D. melanogaster*. This allows further exploration of not only whether invertebrates experience a response comparable to the rewarding or adverse effects of a drug experienced by vertebrate models but also whether these invertebrate models have the receptor systems identified as playing a role in the behavioural response to drugs in vertebrate models.

C. elegans is a soil nematode worm that grows to around 1 mm in adulthood, with a lifespan of 2-3 weeks, that feeds on bacteria such as *Escherichia coli* (Markaki & Tavernarakis, 2010). After first being identified as a suitable metazoan model for neurobiological and developmental biological research (Brenner, 1974), *C. elegans* has become a popular invertebrate model to study the behavioural effects of alcohol consumption as they can

exhibit behavioural changes in response to both acute (Davies et al., 2003) and chronic ethanol consumption (Lee et al., 2009). Acute administration of ethanol was shown to decrease "thrashing" behaviour in a dose-dependent manner where ethanol was hypothesised as entering through the cuticle where equilibrium resulted in a steady-state internal concentration (Mitchell et al., 2007). Ethanol-induced decrease in locomotory behaviour of *C. elegans* was also demonstrated by Davies et al., (2003) in which the study additionally showed that egg laying behaviour was also reduced when ethanol was administered. Chronic ethanol administration in *C. elegans* results in the development of CPP, where prolonged ethanol exposure resulted in the worms showing an attraction to ethanol (Lee et al., 2009). Development of CPP following chronic ethanol consumption has also been shown in *D. melanogaster* (Cadieu et al., 1999), a fruit fly that grows up to 3 mm with a lifespan of 60 to 80 days (Fernández-Moreno et al., 2007) which develops tolerance to alcohol due to its natural diet of fermenting plants (Chakir et al., 1996; Heberlein, 2000).

C. elegans have also been used to study nicotine, which likewise to ethanol, is also absorbed through the cuticle (Smith Jr et al., 2013). Opposite to ethanol, nicotine, at higher concentrations, increased rates of egg laying (Smith, 2011). *C. elegans* response to nicotine also differs from that to ethanol, as locomotory behaviour is dose-dependently increased following nicotine administration (Feng et al., 2006). However, a similarity in the addictive potential of nicotine to ethanol has been observed in *C. elegans,* where they develop CPP for nicotine (Engelmann et al., 2018). Increasing concentrations of nicotine administered to *D. melanogaster* reduced the survival rate from larvae to adult however, larvae that survived and were administered nicotine during developmental stages showed a reduced sensitivity and increased tolerance to nicotine (Skoulakis, 2017).

These results show that invertebrate models have comparable responses in drug administration to other vertebrate models such as rodents and humans.

1.5. Ethanol

Ethanol, also referred to as alcohol, is an organic chemical compound that is colourless, flammable and volatile which can be characterised by its pungent taste and odour (Alam & Tanveer, 2020). Due to its psychoactive properties, alcohol has been classed as one of the most widely used recreational drugs across the world with alcohol consumption becoming a staple in social norms (Sudhinaraset et al., 2016). Alcohol consumption within individuals and populations is driven by how accessible alcohol is which in turn is driven by three factors: availability, affordability, and acceptability (Public Health England, 2016). It is the only psychoactive substance with a dependency-developing risk which significantly impacts global population health that is not regulated with legal binding rulings at a worldwide level (World Health Organisation, 2022a).

1.6. Pharmacology of Ethanol

1.6.1. Pharmacokinetics

How ethanol will affect the body is dependent on the volume of alcohol consumed and the period of ethanol exposure, both of which will depend on ethanol absorption and metabolism in the body (Norberg et al., 2003). Ethanol is absorbed into the bloodstream through the gastrointestinal tract, with the rate of absorption dependent on the volume of alcohol consumed, gastric contents, rate of gastric emptying, smoking and medications (Norberg et al., 2003). Metabolism of ethanol can occur via different pathways dependent on acute or chronic consumption (Figure 1.5.). First pass metabolism of ethanol in the liver involves the oxidation of ethanol into acetaldehyde which is catalysed by alcohol dehydrogenases (ADHs) in the cytosol (Zakhari, 2006). Further oxidation of acetaldehyde into acetate then occurs in the mitochondria and is catalysed by aldehyde dehydrogenase (ALDH) (Zakhari, 2006). Chronic ethanol consumption will induce the microsomal ethanol oxidising system (MEOS), namely cytochrome P450 2E1 (CYP2E1), which will metabolise ethanol into acetaldehyde within the endoplasmic reticulum (Manzo-Avalos & Saavedra-Molina, 2010). This induction of CYP2E1 can be viewed in rat and mouse microsomes; fragments of the endoplasmic reticulum and attached ribosomes which are isolated together when homogenised cells are centrifuged

(Robin et al., 2005). In tissues where there is little ADH activity, like the brain, oxidation of ethanol may be dependent on CYP2E1 and catalase (Zimatkin et al., 2006). Oxidation that occurs via CYP2E1 releases by-products of reactive oxidation species (ROS). Hepatocytes are left more susceptible to the damage caused by metabolism by-products including acetaldehyde and free radicals as oxidation results in hepatocytic cytosol level reduction (Zakhari, 2006). Whilst the role of catalase within ethanol metabolism is seen to be minor, increased levels of hydrogen peroxide (H_2O_2) and therefore increased catalase activity has been shown in rat liver following chronic ethanol consumption (Chen et al., 2021). Acetate leaves the liver and is diffused into the bloodstream where it undergoes metabolism to either carbon dioxide (CO_2) or acetyl CoA (Zakhari, 2006). Ethanol is eliminated from the body at a zero-order elimination rate within a one-compartment model (Jones, 2010), meaning that there is a constant amount of ethanol eliminated from the body over a set time which is independent to the concentration of ethanol in the plasma (Borowy & Ashurst, 2023).

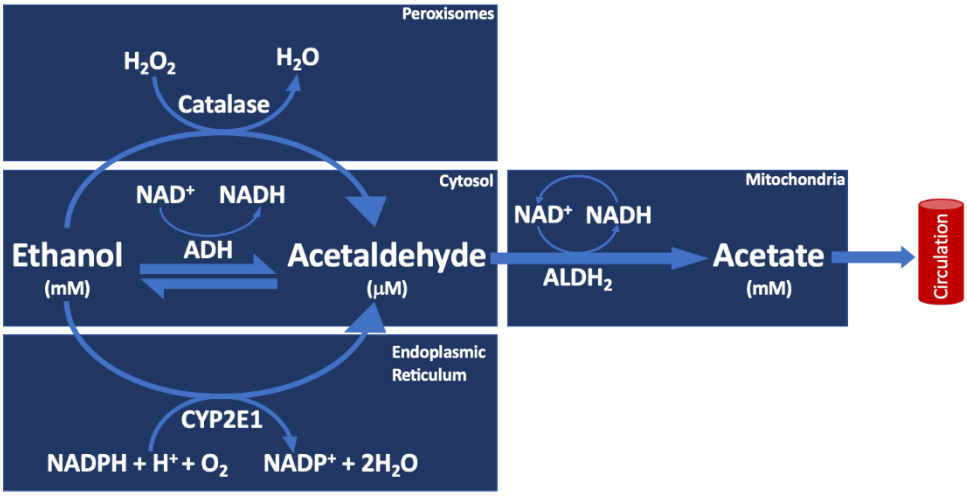


Figure 1.5. Ethanol metabolism. Metabolism of ethanol occurs via different pathways dependent on the site of metabolism within the organelles of the liver cells. The majority of metabolism of ethanol into acetaldehyde occurs via alcohol dehydrogenases (ADH) within the cytosol. Induction of the microsomal ethanol oxidising system (MEOS) also metabolises ethanol into acetaldehyde via CYP2E1 within the endoplasmic reticulum. Catalase is also thought to play a role in ethanol metabolism within the peroxisomes. Acetaldehyde is then metabolised within the mitochondria into acetate, catalysed by aldehyde dehydrogenase (ALDH). Acetate will enter the bloodstream and will be metabolised into CO_2 or acetyl CoA (adapted from Zakhari, (2006)).

1.6.2. Pharmacodynamics

Acute alcohol intoxication can present with symptoms across the body. Disruptions to metabolic pathways can include hypoglycaemia, hypokalaemia, hypocalcaemia, and lactic acidosis. Cardiovascular issues such as tachycardia and peripheral vasodilation can also occur, with the latter contributing to high blood pressure and low body temperatures (Vonghia et al., 2008). Along with respiratory distress such as aspiration due to reduced ciliary clearance, acute alcohol intoxication can also suppress the proinflammatory cytokine response to bacteria, increasing the susceptibility of bacterial infections, such as pneumonia, to the lungs (Happel et al., 2006; Vonghia et al., 2008). Damage to the gastrointestinal system includes nausea and vomiting however can be more severe and include peptic ulcers and pancreatitis, which if left untreated, can be life-threatening (Vonghia et al., 2008).

Presentation of acute alcohol intoxication will be dependent on the blood alcohol concentration (BAC). Whilst a low BAC of <50 mg/dl can result in positive effects such as euphoria, increased sociability, and reduced stress levels, a higher BAC of >200 mg/dl can result in vomiting, hypothermia and amnesia with symptoms worsening to respiratory depression, coma, or death at a BAC of >400 mg/dl (Vonghia et al., 2008).

Low daily alcohol intake has been shown to lead to reduced risks of cardiovascular disease (Gaziano et al., 2000) however chronic alcohol misuse can have more serious consequences such as liver disease, a risk of dementia, bone damage and cancer (Callaci et al., 2009; Grønbaek, 2009; Poschl & Seitz, 2004).

Alcohol intake can also disrupt neurotransmitter systems and it has been suggested that the symptoms of intoxication are caused by the changes to neurotransmitter receptor activity (Tambour & Quertemont, 2006). Two of the main neurotransmitter receptor pathways altered when exposed to alcohol are the GABAergic and glutamatergic receptor pathways: whilst acute ethanol administration upregulates the GABA receptors (Davies, 2003), it downregulates the *N*-methyl-d-aspartate (NMDA) receptors (the main receptors in the glutamatergic pathway) (Krystal et al., 2003). Chronic alcohol exposure has been suggested to

have the opposite effect by downregulating the GABA receptors and upregulating the NMDA receptors (Devaud & Alele, 2004).

1.7. Alcohol misuse

Alcohol misuse is responsible for 3 million deaths a year (5.3% of all deaths) and contributes to 5.1% of disability-adjusted life years (DALYS). Alcohol use disorders (AUD) have a higher mortality rate than diabetes, HIV/AIDS, and tuberculosis (World Health Organization, 2018). In 2020, in the United Kingdom, there was a significant increase of 19% in deaths as having alcohol-specific causes compared to 2019 and rates of alcohol-specific deaths in males were doubled compared to females (Office for National Statistics, 2021). In 2016, alcohol misuse was shown to lower life expectancy with the average age of patients dying from an alcohol-related cause was 54.3 years compared to the average age of people dying from all causes at 77.6 years (Public Health England, 2016). AUDs alone (with no other drug treatment) make up one of the largest proportion of patients in substance use treatment in England, second to opiate misuse, and deaths of patients in AUD treatment increased by 44% in 2020/21 compared to 2019/2020 (this, however, may have attributed to the COVID-19 pandemic) (Office for Health Improvement & Disparities, 2021). Responsible for 77.8% of alcohol-specific deaths, alcoholic liver disease was the main cause of alcohol-specific related deaths in the United Kingdom in 2020 (Office for National Statistics, 2021).

Alcohol dependence, a type of AUD, is defined by the World Health Organisation as “a need for repeated doses of [ethanol] to feel good or to avoid feeling bad” (World Health Organisation, 1994). It is characterised by “cognitive, behavioural and psychologic symptoms” such as cravings, an obsession with alcohol and constant drinking despite any adverse effects, suggesting a lack of control regarding alcohol consumption (National Institute for Health and Care Excellence, 2011; World Health Organisation, 1994). Dependency is also characterised by the presence of alcohol withdrawal syndrome when there is a sudden cessation in alcohol consumption. This can present with symptoms such as tremors, seizures, hallucinations and insomnia (Diamond & Messing, 1994). These symptoms can often play a role within negative reinforcement, where an individual will revert to drinking alcohol to avoid or escape the symptoms of withdrawal (Jesse et al., 2016). Animal models, such as rats, who were

chronically consuming ethanol, have demonstrated increased alcohol seeking behaviour following a period of alcohol abstinence (Sinclair & Senter, 1967).

Since 1970, deaths due to alcohol-related liver disease have increased by 400% (Public Health England, 2016). Alcoholic liver disease can present in many forms, starting from fatty liver and developing into more serious complications such as alcoholic hepatitis (liver inflammation), cirrhosis (scarred liver tissue) and primary liver cancer. Over 90% of chronic alcohol drinkers will develop a fatty liver following the early stages of their chronic consumption however only 30% will suffer from the more critical complications, such as liver cirrhosis (Gao & Bataller, 2011).

Steatosis, also known as alcoholic fatty liver, is the first pathological symptom to develop following ethanol consumption. This can be developed following acute and chronic ethanol exposure. Whilst the pathophysiological pathway responsible for alcoholic fatty liver has been thought to be due to inhibition of fatty acid β-oxidation within the mitochondria, newer pathways of inhibiting oxidation of fatty acids have been suggested: inhibition of peroxisome proliferator-activated receptor α (PPARα), which is responsible in controlling the response of liver cells to fatty acids (Galli et al., 2001), and inhibition of AMP-activated protein kinase (AMPK), which is responsible for regulating metabolic pathways during cellular stress (such as oxidative stress) (Sozio & Crabb, 2008). There is a dose-dependent increase in risk of developing liver cirrhosis following daily alcohol consumption, with the risk found to be higher in women than in men (Becker et al., 1996).

1.8. Ethanol tolerance

Alcohol tolerance is defined as “a loss of efficacy with repeated [ethanol] exposure” meaning that to experience the same effect following a certain volume of ethanol consumption, the volume of ethanol consumed would have to be increased (Bespalov et al., 2016). Individuals who are pre-dependent but have a high likelihood of developing tolerance to ethanol may be more likely to misuse alcohol and develop alcohol dependence (Wallace et al., 2007). Ethanol tolerance can have an impact on behaviour, known as behavioural tolerance, or it can impact cellular processes, known as physiological tolerance. Tolerance can be divided into two

categories, depending on the timeframe of ethanol consumption: acute tolerance and chronic tolerance (Chandler et al., 1998). Acute functional tolerance (AFT) covers the short-term behavioural changes following a single-dose acute ethanol exposure (Comley & Dry, 2020). To observe AFT, the BAC of the experimental subject is the dose, and the behavioural impact of the same BAC is observed over time. AFT was first described by Mellanby (1919) where the response to the same BAC over time demonstrated a two-limbed curve, highlighting a rapid increase from baseline to peak response (the ascending limb) and a slower decrease from peak response back to baseline (Figure 1.6.).

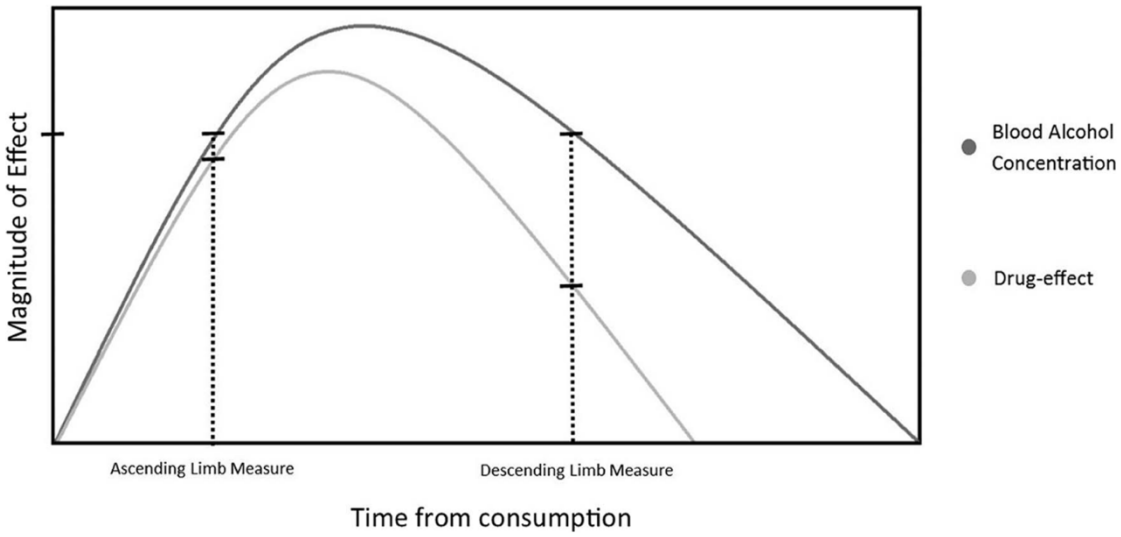


Figure 1.6. Magnitude of effect when comparing blood alcohol concentration (BAC) versus drug-effect of ethanol over a single exposure period. This graph demonstrates the Mellanby effect where the ascending limb highlights a rapid increase in response to drug-effect compared to a decreased response to drug-effect on the descending limb at the same BAC (taken from Comley & Dry, (2020a)).

1.9. Ethanol toxicity

Toxicity as a result of ethanol consumption occurs at a cellular level. An altered hepatocytic mitochondrial structure is one of the first signs of alcohol consumption, whereby the mitochondria look larger and warped with damaged cristae (Kiessling & Tobé, 1964). Animal models, such as rats, will often also present with a fatty liver, as mentioned in 1.2.2. (Souza et al., 2015). Pathogenesis of alcoholic liver disease is associated with an increase in the peroxidation of lipids in the liver, leading to fatty liver, which can be attributed to the

production of free radicals and oxidative stress (Kalish & Di Luzio, 1966). Oxidative stress can also lead to ethanol-induced neurological damage (Haorah et al., 2008).

Free radicals are highly unstable atoms, molecules or compounds that will react with other molecules or free radicals to become stable. Reactive oxygen species (ROS) are free radicals that contain oxygen; the primary ROS are superoxide ($O_2^{\bullet-}$), peroxide ($O_2^{=}$) and hydroxyl ($^{\bullet}OH$). ROS can be formed during respiration where oxidisation of NADH to NAD^+ results in an electron (e^-) and proton (H^+) being released from NADH. This electron will then be carried down the electron transport chain (ETC) to bind with molecular O_2. In normal physiological conditions, the addition of 4 e^- and 4 H^+ to an O_2 molecule will generate water (H_2O) however, in the pathophysiological state, e^- leakage can occur and univalent reduction of O_2, when only one electron is added to the O_2 molecule, results in the formation of $O_2^{\bullet-}$ (Juan et al., 2021). Due to their unstable nature, most ROS are unable to damage cells as they react quickly with any free electrons and protons and are converted into water (Wu & Cederbaum, 2003). As ROS are naturally formed during metabolic processes, cells will use antioxidants to either stop ROS forming or to reduce their toxicity (Yu, 1994).

One of the first studies linking ethanol consumption and the formation of free radicals demonstrated that administration of antioxidants prevented fatty liver caused by ethanol (Di Luzio & Poggi, 1963). This led to a multitude of studies investigating lipid peroxidation caused by ethanol-induced free radical formation in rats' hearts and livers (Haorah et al., 2008; Reinke et al., 1987; Rouach et al., 1997), suggesting that alcohol has pro-oxidant mechanisms. Oxidative stress induced by alcohol consumption includes the involvement of the metabolic enzymes ADH, ALDH, catalase and CYP2E1, increasing the formation of ROS such as $O_2^{\bullet-}$ and H_2O_2 (Albano, 2006). ADH and ALDH in ethanol's metabolic processes involve the reduction of NAD^+ to NADH meaning that alcohol consumption results in an imbalance of the cellular NAD^+/NADH ratio (Wu & Cederbaum, 2003). Excess NADH produced by this metabolism is used by the respiratory chain and therefore a continuous source of NADH can contribute to a continuous generation of ROS (Das & Vasudevan, 2007; Mira et al., 1995). Further studies have demonstrated ethanol further intensifies oxidative stress by diminishing the protective antioxidative mechanisms (Azzalis et al., 1995; Fernández-Checa et al., 1991). A higher rate of ROS formation compared to a lower rate of ROS removal and repair of damaged cells will then

result in oxidative stress. Following chronic ethanol administration, rat liver microsomes have shown increased $O_2^{\bullet -}$ and H_2O_2 production compared to controls (Boveris et al., 1983; Ekström & Ingelman-Sundberg, 1989) suggesting the role of both catalase and the CYP2E1-mediated MEOS metabolism of ethanol. Ethanol-derived free radicals, such as 1-hydroxyethyl, have also been shown to be formed by MEOS (Albano et al., 1987).

Ethanol consumption can also dysregulate neurotransmitter systems. Teplova et al., (2017) demonstrated that following chronic ethanol consumption, mitochondrial glutamate dehydrogenase (GLDH) protein expression and activity increased in the liver mitochondria of alcohol dependent rats. The same study also highlighted that when glutamate was present, the liver mitochondria of these rats released more ROS, superoxide anion and H_2O_2 compared to the mitochondria of control rats (Teplova et al., 2017). These results demonstrate that the the increase in glutamate release and upregulation of NMDA receptors can contribute to the hepatotoxicity seen following chronic ethanol consumption. However, an earlier study by Kravos & Malesic (2010) demonstrated that GLDH activity decreased in human leukocytes following alcohol consumption but the increased following a break in alcohol consumption: up to 48 hours following the last alcohol intake, there was, on average, a 32% increase in human leukocyte GLDH activity. This further suggests that glutamate release following alcohol consumption has long term impacts on cellular toxicity.

Other oxidative enzymes such as xanthine oxidase, following ethanol exposure, will also be altered to contribute to the increased formation of ROS. Xanthine dehydrogenase is a precursor for xanthine oxidase. Xanthine oxidase's normal role as a dehydrogenase will catalyse the reduction of NAD^+ to NADH, contributing to the NAD^+/NADH ratio imbalance (Kostić et al., 2015). Ethanol exposure has been suggested to increase the production of xanthine oxidase from xanthine dehydrogenase, therefore increasing NADH production and ROS formation (Sultatos, 1988).

Heat shock proteins (Hsps) are molecular chaperones that are important for cell development and survival (Miller & Fort, 2018). Hsps can be classified as either small ATP-independent Hsps, which have a molecular mass between 8 to 28 kDa, and large ATP-dependent Hsps, which have a molecular mass between 40 to 105 kDa (Miller & Fort, 2018). Hsps are highly conserved

from humans to bacteria (Dubey et al., 2015) and were first described after heat-shock resulted in their increased production in *drosophila* (Ritossa, 1962). Since then, their physiological functions in folding, transport and repair or breakdown of proteins have been determined (Dubey et al., 2015). Increased expression of Hsps following pathological stimuli, such as oxidative stress (Calabrese et al., 2010), cellular apoptosis (Choi et al., 2014) and neuroinflammation (Dukay et al., 2019) suggests that they exhibit a wide range of protective roles. Heat shock factor 1 (HSF1) is a transcription factor for Hsp60, which is involved in mitochondrial regulation (Cheng et al., 1989), Hsp70, which is involved in maintaining the structure of proteins by regulating their folding (Mayer & Bukau, 2005) and Hsp90, which is involved in cell cycle regulation and also in activating the adaptive immune system (Hoter et al., 2018). Activation of HSF1 occurs when cellular stress causes misfolding of cellular proteins; this results in Hsps dissociating from HSF1 and binding to the misfolded proteins (Prahlad & Morimoto, 2008).

HSF1 activation has been shown to increase following ethanol-induced oxidative stress (Pignataro et al., 2007), meaning that Hsps may have a role as a marker for ethanol toxicity. *In vitro* studies have demonstrated that an increase in HSF1 binding increases expression of Hsp70 increases following acute ethanol exposure in honeybee brains (Hranitz et al., 2010) and more recently in human monocytes, with ethanol induced Hsp70 increase contributes to inhibition of pro-inflammatory cytokines (Mandrekar et al., 2008; Muralidharan et al., 2014). Mandrekar et al., (2008) demonstrated that on human monocytes, similar increases were seen for Hsp90 expression, which was also increased in rat liver cells following ethanol exposure (Ikeyama et al., 2001). *In vivo* studies have demonstrated that in patients who chronically consume alcohol, Hsp70 circulatory serum levels are significantly increased however when severe forms of alcoholic fatty liver disease (AFLD) develop, the expression of Hsp70 appears to be downregulated although the mechanism as to why is still unclear (Qu et al., 2015). Hsp90 acetylation was shown to increase following the metabolism of ethanol by MEOS in rats and mice (Yang et al., 2021). Currently, there are no studies which link an increase in Hsp60 expression with alcohol exposure.

Cytochrome C (Cyt*c*) is a mitochondrial protein that, similar to Hsps, is involved in cell cycle regulation, more specifically, respiration and apoptosis (Hüttemann et al., 2011). It also has

antioxidant properties as it is involved the removal of ROS where it will interact with $O_2^{\bullet-}$, removing the electron to generate O_2 (Pereverzev et al., 2003). These properties of Cyt*c* suggest its suitability as a possible toxicity marker.

Due to increased Cyt*c* activity during oxidative stress, increased release of Cyt*c* has been demonstrated during ethanol metabolism (Mira et al., 1995) and following chronic administration of ethanol (Graw et al., 2015) in rats lungs and spleen. When inducing apoptosis via ethanol administration, it was shown that an influx of Ca^{2+} into the mitochondria mediated almost all of the Cyt*c* in the mitochondria was release after 24h (Nakayama et al., 2001).

1.10. GABAergic System

First identified in plants, mammals' brains, and animals, then more recently in bacteria and fungi, Gamma-aminobutyric acid (GABA) is one of the main inhibitory neurotransmitters within the central nervous system (CNS), with 60-75% of synapses being GABAergic (Hepsomali et al., 2020; Schwartz, 1988). GABA mediates $GABA_A$ receptors, ligand-gated ion channels which were originally identified due to their activation by GABA, and $GABA_B$ receptors, G-protein coupled receptors (Olsen & Sieghart, 2008). Disruptions to the GABAergic systems can result in neurological diseases including Alzheimer's disease, Huntington's disease, Parkinson's disease, and schizophrenia (Wong et al., 2003a) as well as mood disorders such as major depressive disorder and bipolar disorder (Krystal et al., 2002) making these systems targets for treatment.

As ethanol interacts with the CNS, GABAergic systems have been identified as a target pathway through which ethanol is thought exert its depressant effect (Figure 1.7; Förstera et al., (2016)). Whilst the interactions of ethanol are different with the $GABA_A$ receptor compared to the $GABA_B$ receptor, both receptors are key targets in the understanding of ethanol's mechanism of action and therefore are important to consider for alcohol abuse treatment options.

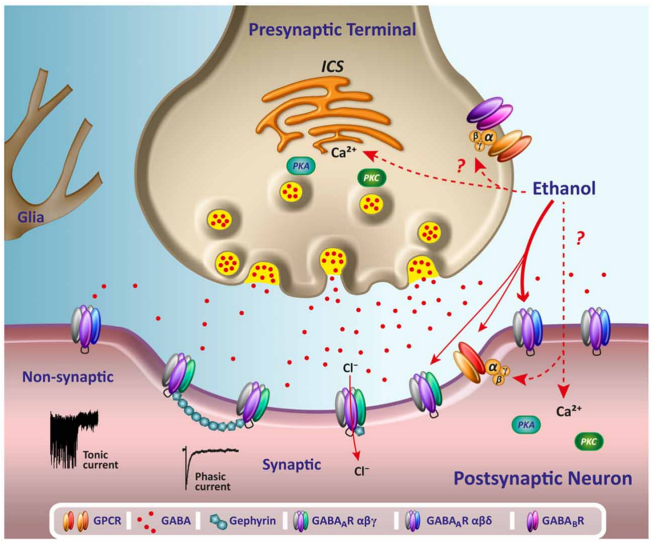


Figure 1.7.Illustration of how acute ethanol administration affects GABAergic pathways. This diagram highlights how both the GABAA and GABAB receptor pathways are affected when ethanol enters the body (taken from Förstera et al., (2016)).

1.11. $GABA_A$ Receptors

$GABA_A$ receptors belong to the Cys-loop pentameric ligand-gated ion channel (LGIC) superfamily, which also includes nicotinic acetylcholine receptors (nAChRs), ionotropic serotonin receptors and inhibitory glycine receptors (Olsen & Sieghart, 2008). They are heteropentamers, made up of 5 of the following subunits: α1-6, β1-3, γ1-3, δ, ε, θ or π (Olsen & Sieghart, 2008). Due to heterogeneity of the receptor structure, the pharmacological profile of the $GABA_A$ receptor will be determined by the subunits present. Within mammals, the most common subunit composition of the $GABA_A$ receptor within the brain consists of two α1, two β2 and one γ2 subunits (43%) (Davies, 2003; Förstera et al., 2016; Pirker et al., 2000). Within each subunit, there is an extracellular, hydrophilic N-terminal domain containing a ligand-recognition site, thought to be made up of "loops" of amino acids, to which the neurotransmitter will bind. Four α-helix transmembrane domains cover the length of the membrane; the second of these subunit domains make up the lining of the ion channel. An extracellular C-terminal domain is present at the end of the subunit and is shorter in comparison to the rest of the subunit. The binding sites on the N-terminal make up pockets to which the ligand can bind; there are 2 pockets where the β subunit A, B, C "loops" meet the α subunit D, E, F "loops" which make up 2 GABA binding sites. All five subunits are

arranged in a circle, creating an ion pore in the middle which opens when a ligand binds to the N-terminal. The ion pore within $GABA_A$ receptors will conduct chloride ions (Cl^-) which will be transported down an electrochemical gradient. Once GABA activates the receptor, this will trigger an influx of Cl^- within the postsynaptic cell, lowering the membrane potential and delaying the firing of the action potential, hence why GABA is categorised as an inhibitory neurotransmitter (Davies, 2003; Olsen & Sieghart, 2008). Ernst et al., (2005) demonstrated that in addition to the extracellular pockets making up the binding sites, $GABA_A$ receptors have extra cavities within the transmembrane domain and within each subunit's four α-helices. It was proposed that these cavities may not only allow for the conformational change in receptors but may also be used as allosteric binding sites for drugs. If a drug was to bind to the cavity, this could cause a conformational change within the receptor, increasing or decreasing the GABA-induced Cl^- influx (Ernst et al., 2005). One of these cavities, when bound to alcohol in high concentrations, was found to cause allosteric modulation and potentiate $GABA_A$ receptor function (Mihic et al., 1997).

Early behavioural, biochemical, and electrophysiological studies led to the hypothesis that acute exposure to ethanol potentiates the $GABA_A$ receptor in a similar way to benzodiazepines and barbiturates. Initial behavioural studies showed that ethanol-induced intoxication was potentiated following administration of GABAmimetic drugs, such as muscimol and reduced following administration of GABA antagonists, such as bicuculline (Frye & Breese, 1982); Breese et al., (1984) demonstrated that the administration of bicuculline methiodide to rats during ethanol-induced depression caused a significant increase in movement. Givens & Breese (1990) demonstrated a similar result when demonstrating that a microinjection of bicuculline reduced the length of sedation induced by ethanol. A study completed by Suzdak & Paul (1987) first demonstrated ethanol induced a 260% increase in chloride ion uptake through a $GABA_A$ receptor in mice brains. Using the phasic and tonic currents that are used to send GABAergic signals to granule cells, electrophysiological techniques illustrated an increase in the occurrence of phasic currents and the level of tonic currents following 50 mM ethanol administration (Carta et al., 2004).

This level of ethanol-induced potentiation is thought to be affected by the site of action and the $GABA_A$ receptor subunits (and their variations) present at that site. At concentrations of

50 mM ethanol and above, it has been demonstrated that receptors containing the γ subunit will show significant potentiation (Ueno et al., 2001) and furthermore, that the γ2L variant of the γ2 subunit must be present (Wafford & Whiting, 1992). Other studies, however, have been unsuccessful in replicating any impact of the variant of the γ2 subunit on ethanol induced $GABA_A$ receptor potentiation (Mihic et al., 1994; Zhai et al., 1998). Alternatively, following findings that substituting the γ subunit in 2α-2β-1γ with a δ subunit can lead to an increase in the affinity of GABA to the receptor by up to 50-fold (Brown et al., 2002; Saxena & Macdonald, 1994; Wohlfarth et al., 2002), Sundstrom-Poromaa et al., 2002 have suggested that the less expressed α4βδ $GABA_A$ receptor may show increased sensitivity to lower concentrations of ethanol compared to other $GABA_A$ receptor subtypes. It was further suggested by Wallner et al., (2003) that the β3 subunit in the α4βδ $GABA_A$ receptor composition makes the receptor 10 times more sensitive to ethanol than if a β2 subunit was present however this has been unable to be replicated (Sanchis-Segura et al., 2007).

1.12. Bicuculline

Bicuculline is a competitive and selective $GABA_A$ receptor antagonist (Johnston, 2013) derived from a variety of plants including *Dicentra cucularia* and *Adlumia* (Srivastava et al., 2011). The pharmacokinetics of bicuculline are not well described in literature however at a physiological pH, bicuculline can be converted to bicucine (Olsen et al., 1975) and can also reach the CNS dose-dependently (Yamazaki et al., 2020). Whilst bicuculline competes with GABA to bind to the receptor, it has been suggested that bicuculline has two binding sites and therefore acting as an allosteric ligand (Ueno et al., 1997), reducing $GABA_A$ receptor activity by shortening the length of time that the Cl^- channels are open (Macdonald et al., 1989). Bicuculline's antagonistic effects are not impacted by the $GABA_A$ subunits present in the receptor. Bicuculline acts a convulsant (Johnston, 2013) and has been administered alongside ethanol to observe its anticonvulsant effect (Zhuk et al., 2001). Bicuculline's antagonistic properties have also been a valuable tool in observing the role of the $GABA_A$ receptor pathway in the mechanism of action of ethanol in behavioural studies, where bicuculline reduced ethanol self-administration (Kemppainen et al., 2012) and ethanol-induced locomotory activity (Chester & Cunningham, 1999). Ethanol-induced cardiovascular changes such as low blood pressure and heart rate were prevented with the administration of bicuculline (Phelix et al.,

1999). These studies further empathise the hypothesised role of the $GABA_A$ receptor pathway for ethanol's mechanism of action.

1.13. $GABA_B$ Receptors

$GABA_B$ receptors are G-protein coupled receptors (GPCRs) (Terunuma, 2018) formed of two subunits: R1 and R2 (Jones et al., 1998). R1 and R2 subunits have similar structures made up of a Venus Flytrap Domain (VFT), which is the extracellular N-terminal that provides a binding site for GABA, followed by 7 α-helix transmembrane domains which end with the intracellular C-terminal which is coupled with G-proteins from the Gαi/o family (Terunuma, 2018). R1 and R2 subunits differ by their function; whereas orthosteric binding of ligands, such as GABA, to the VFT will occur only on the R1 subunit, coupling of the G-protein will only occur via the R2 subunit (Figure 1.8.; Margeta-Mitrovic et al., 2001).

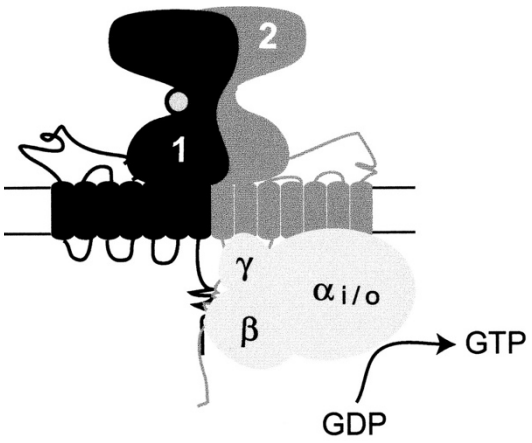


Figure 1.8. Structure of the $GABA_B$ receptor. The heterodimer structure is vital for $GABA_B$ receptor activity, allowing for binding of the ligand at R1 (black) and signalling mediated by G-protein at R2 (grey). Taken from Margeta-Mitrovic et al., (2001).

$GABA_B$ receptor activation by GABA binding mediates synaptic inhibition through G-protein uncoupling, where the G proteins will dissociate from the receptor into their Gα and Gβγ subunits which will interact with secondary messengers (Geng et al., 2013). G-protein mediated signalling can affect three pathways: G-protein activated inwardly rectifying K^+ (GIRK) channels and voltage-gated Ca^{2+} (Ca_V) channels, both impacted by Gβγ or cyclic AMP

(cAMP) production by adenylyl cyclase, impacted by Gα (Figure 1.9.) (Bettler et al., 2004). The potentiation or inhibition of these pathways can in turn result in inhibition of neurotransmitter release and increase of excitatory neuronal activity (Bettler et al., 2004). Activation of GIRK channels by Gβγ will cause an efflux of K^+ outside the synapse. This results in membrane hyperpolarisation, known as a slow inhibitory postsynaptic potential (IPSP) and inhibition of the firing of action potentials (Lüscher et al., 1997). Gα has also been shown to interact with the intracellular domains of GIRK channels to activate the channel (Clancy et al., 2005). Gβγ inhibits both presynaptic and postsynaptic Ca_V channels from opening meaning that membrane depolarisation due to the calcium influx into the neuron is stopped (Chalifoux & Carter, 2011; Harayama et al., 1998). Inhibition of adenylyl cyclase by Gα results in a reduction of cAMP production (Hill, 1985; Holopainen et al., 1992). Whilst the significance of limiting adenylyl cyclase activity is not fully understood, it is thought that by reducing cAMP production, neurotransmitter release is reduced due to limited synaptic vesicle release, also mediated by Ca_V channels (Sakaba & Neher, 2003).

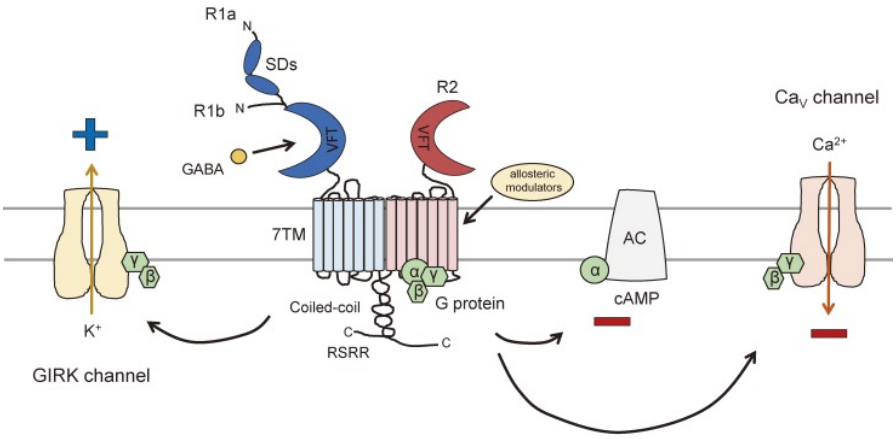


Figure 1.9. $GABA_B$ receptor pathways. G-protein uncoupling due to GABAB receptor activation can activate three different pathways: GIRK channels, CaV channels and adenylyl cyclase (taken from Bettler et al., 2004).

$GABA_B$ receptors are thought to play an opposite "anti-alcohol" role to $GABA_A$ receptors in ethanol consumption. Behavioural studies have demonstrated that activation of $GABA_B$ receptors via administration of $GABA_B$ agonists, such as baclofen, dose-dependently reduce self-administration of alcohol in alcohol-dependent rats (Walker & Koob, 2007) and humans (Addolorato, 2002). Binge-drinking of alcohol in mice was reduced (Moore & Boehm, 2009) as

well as CPP for alcohol (Bechtholt & Cunningham, 2005) and locomotory behaviour induced by ethanol (Boehm et al., 2002). Furthermore, double-blind, randomized placebo-controlled trials have shown that baclofen treatment reduced alcohol cravings and led to reduced or no alcohol consumption in alcoholics (Addolorato, 2002; Morley et al., 2018). One of these trials also demonstrated that side effects due to alcohol withdrawal, such as anxiety, were also reduced (Addolorato, 2002) which was also seen in preclinic studies alcohol-dependent rats (Knapp et al., 2007). Allosteric modulation of $GABA_B$ receptors by positive allosteric modulators (PAM) have also shown to reduce daily alcohol consumption in alcohol-dependent rats (Loi et al., 2013). PR studies showed that the BP for alcohol was reduced in rats following $GABA_B$ agonist and PAM treatment, suggesting that $GABA_B$ activation reduces the reinforcing properties of alcohol (Maccioni et al., 2012; Walker & Koob, 2007).

1.14. Baclofen

Baclofen is a $GABA_B$ receptor agonist that can be administered orally or intrathecally, should oral administration have no effect (Agabio & Colombo, 2014). Baclofen undergoes rapid absorption in the small intestine however has a limited metabolism of 15% in the liver, with 80% of orally administered baclofen being renally excreted (Wuis et al., 1989). Originally developed as an antiepileptic drug, baclofen is clinically used in the treatment of spinal cord issues such as multiple sclerosis (Romito et al., 2021). Both pre- and post-synaptic $GABA_B$ receptors are acted on by baclofen, where it inhibits polysynaptic reflexes via hyperpolarisation of the membrane to reduce muscle stiffness (Allerton et al., 1989). Clinical studies observing the role of baclofen and alcohol consumption and withdrawal (Addolorato, 2002; Morley et al., 2018) have led to the off-label use of baclofen in treating alcohol use disorders. As the mesolimbic dopaminergic system can mediate alcohol-seeking and -taking behaviour, inhibition of this pathway by $GABA_B$ receptor activation during alcohol consumption will lead to a decrease in the release of dopamine, reducing the positive reinforcing sensation experienced normally during alcohol intake (Agabio & Colombo, 2014). Adverse effects seen with baclofen treatment in patients with AUD include headaches, vertigo and tiredness and have been reported as being mild, with clinical trials reporting little to no patients stopping baclofen treatment due to adverse effects (Addolorato et al., 2011; Garbutt et al., 2010).

1.15. Nicotine

Nicotine, like ethanol, is a psychoactive substance that, in its pure form, is a clear and odourful liquid (Mishra et al., 2015). Tobacco, the plant from which nicotine was first isolated by Posselt & Reimann, (1828), accounts for more than 8 million deaths worldwide and can kill up to half of those who use it (World Health Organisation, 2022b). Whilst smoking cigarettes is the most common form of tobacco consumption, other ways of using tobacco include cigars, waterpipe tobacco and pipe tobacco (World Health Organisation, 2022b). Unlike ethanol, however, tobacco is highly regulated with 170 countries signing the "WHO Framework Convention on Tobacco Control (WHO FCTC)" treaty (WHO Framework Convention on Tobacco Control (WHO FCTC), 2003).

1.16. Pharmacology of Nicotine

1.16.1. Pharmacokinetics

Absorption of nicotine will be dependent on method of administration and the pH of its surroundings. When inhaling tobacco smoke, depending on how the tobacco has been "cured", the smoke will either be more acidic or more basic: in more acidic environments, nicotine will ionise due its basic pH of 8 and therefore less absorption will occur via the mouth compared to the inhalation of more basic tobacco smoke (Armitage & Turner, 1970; Benowitz et al., 2009). Rapid absorption of nicotine occurs through the lungs at the alveoli, facilitated by the alveoli's large surface area and basic pH of the lungs (Benowitz et al., 2009) and can also occur via the gastrointestinal tract (Wu & Cho, 2004). Oral nicotine products such as chewing tobacco, nicotine gum, sublingual tablets and lozenges and nasal nicotine products such as nasal spray are "buffered" to ensure a basic pH and to therefore allow buccal absorption with a bioavailability of 40% (Benowitz et al., 2009; Gisleskog et al., 2021). Transdermal absorption of nicotine is extremely potent and rapid, showing a bioavailability of 76% (Gisleskog et al., 2021). Nicotine will be distributed at a steady-state volume of 2.6 L/kg via the bloodstream where 69% will undergo ionisation, 31% will remain unionised and less than 5% will bind to plasma proteins (Benowitz et al., 1982). Again, the method of administration will affect the distribution of nicotine: when smoking, nicotine will be rapidly

distributed to the pulmonary venous circulation where it then moves on to the arterial circulation and the brain (Benowitz et al., 2009). Nicotine can also be distributed to adipose tissue (Won et al., 2014). Through intravenous administration, there is an immediate distribution of nicotine to the brain (Aoki et al., 2020). Nicotine metabolism occurs via a variety of pathways (Figure 1.10.) in the liver and can be divided into phase I and phase II (Mishra et al., 2015). Phase I nicotine metabolism covers the role of oxidases within the liver. This includes the CYP2A6-mediated pathway, which is responsible for 70-80% of nicotine metabolism, where metabolism will produce a nicotine-Δ1' (5')-iminium ion which will be further metabolised by aldehyde oxidase to produce the common nicotine metabolite, cotinine (Benowitz et al., 2009; von Weymarn et al., 2006). Another oxidative pathway, responsible for 4-7% of nicotine metabolism to nicotine N'-oxide is via a flavin-containing monooxygenase 3 (FMO3) (Park et al., 1993). Phase II nicotine metabolism covers the role of N'-and O'-glucuronidation (Mishra et al., 2015). This includes the use of uridine diphosphate-glucuronosyltransferase (UGT) enzymes which are responsible for 3-5% of nicotine metabolism to nicotine glucuronide (Benowitz et al., 2009).

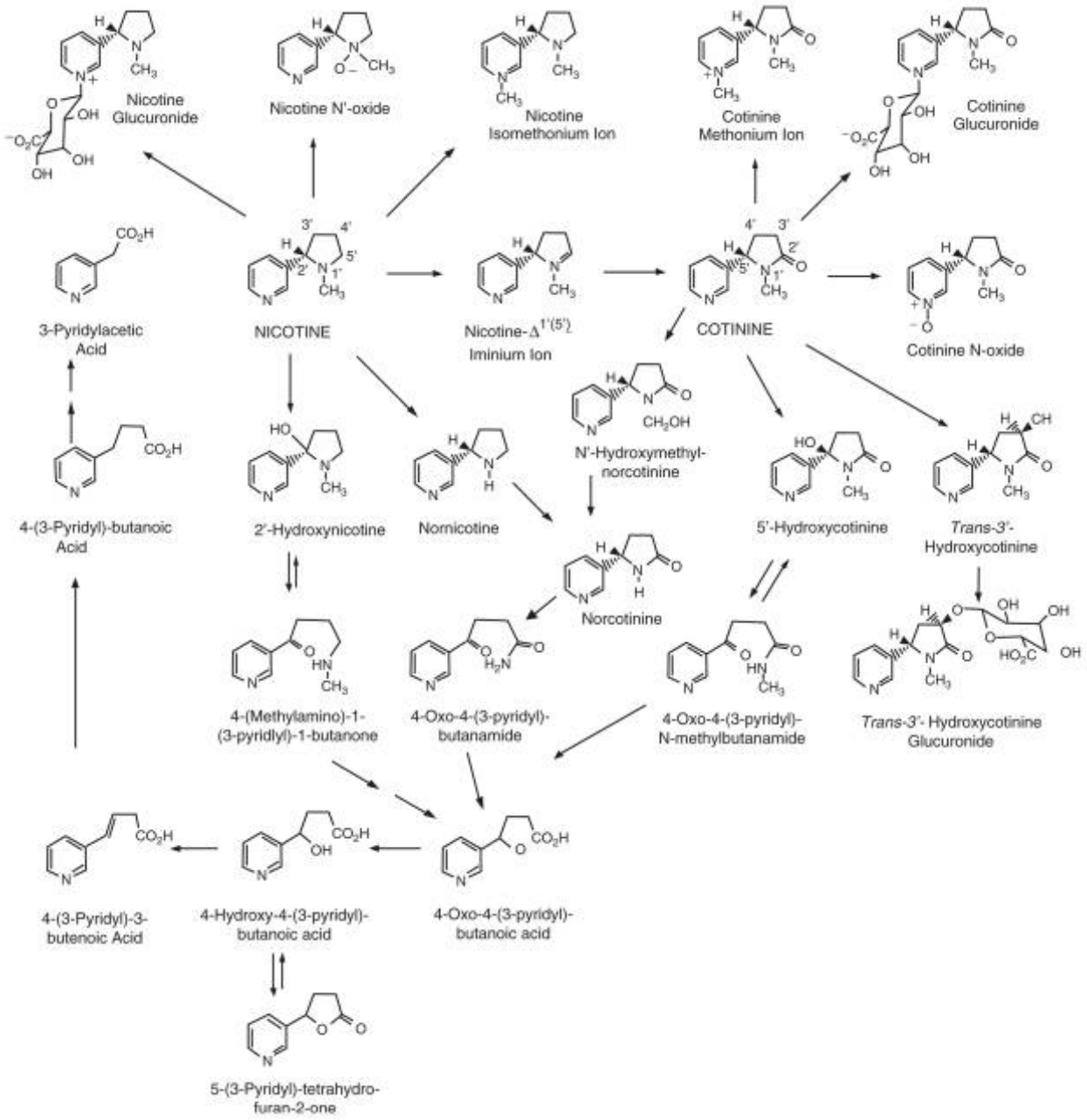


Figure 1.10. Metabolism of nicotine. Metabolism of nicotine can result in the production of a variety of metabolites including cotinine and nicotine N'-oxide (taken from Hukkanen et al., (2005).

Most of the nicotine excretion will occur via its metabolites through urine (Byrd et al., 1992), faeces (Hukkanen et al., 2005), bile (Seaton et al., 1993) and sweat (Concheiro et al., 2011) however 5-10% of nicotine can remain unchanged, and this will be excreted through the renal system dependent on the pH of the urine (Molander, 2000).

1.16.2. Pharmacodynamics

Nicotine acts as a stimulant across various physiological systems by activating the sympathetic nervous system. Psychoactive responses of nicotine include feeling alert and relaxed, improving concentration and performance hence why many smokers will use smoking to regulate their mood during stressful times to lower their anxiety (Benowitz, 2009). Applying nicotine directly on the skin can result in gastrointestinal issues such as irritation and burning in the mouth and throat, vomiting and diarrhoea (Smith et al., 1992). Administered intravenously or inhaled, the gastrointestinal effects of nicotine include increased ulcer formation due to a decreased gastric mucosal blood flow, increased secretions of pepsinogen and reduced epidermal growth factor (EGF) and prostaglandin levels (which provide protective mechanisms against the formation of ulcers) (Wu & Cho, 2004). It can also increase metabolic rates, therefore lowering appetite and causing weight loss (Golli et al., 2016; Perkins, 1992). Circulatory effects of nicotine administration include an increased heart rate and blood pressure, skin, and blood vessel constriction, which can lead to cold fingertips, and skeletal muscle blood vessel dilation (Benowitz & Burbank, 2016) as well as heart failure due to an enlarged heart that can be caused by heart tissue remodelling (van Berlo et al., 2013). Nicotine also results in an increase in the release of catecholamines which can lead to an increase in both atrial and ventricular fibrillation, putting nicotine-users at an increased risk of sudden cardiac death (Benowitz & Burbank, 2016). Cigarette smoke has been widely linked with the development of cancer and whilst the carcinogenic effects of nicotine have had contradictory findings, recent studies carried out both *in vitro* and *in vivo* have demonstrated that nicotine may assist in the development of cancer. Genotoxic effects of nicotine have been demonstrated in both *Escherichia colipol* (Riebe et al., 1982) and human lymphocytes (Ginzkey et al., 2013; Trivedi et al., 1990) and nicotine's role in cell proliferation and tumour formation has been demonstrated in both endothelial cells (Villablanca, 1998) and rodents models (Waldum et al., 1996).

1.17. Cholinergic System

Acetylcholine (ACh), a rapid, excitatory neurotransmitter and neuromodulator, found in both the peripheral nervous system (PNS) and CNS respectively, mediates its effects through two

receptor types as part of the cholinergic system: muscarinic acetylcholine receptors (mAChRs) or nicotinic acetylcholine receptors (nAChRs) (Picciotto et al., 2012). ACh is endogenously produced following the acetylation of choline with acetyl-coenzyme A (acetyl-CoA) and is responsible for regulating brain functions such as response to sensory stimuli (Minces et al., 2017), motivation (Marche et al., 2017) and learning (Atallah et al., 2014). Pathophysiology of the cholinergic system can result in the development of motor neurone diseases such as Parkinson's (Ztaou et al., 2016), Alzheimer's (Hampel et al., 2018) and Huntington's (Smith et al., 2006) diseases as well as neuropsychiatric disorders such as depression (Cheng et al., 2019) and autism spectrum disorders (Nagy et al., 2017).

1.18. Muscarinic acetylcholine receptors

mAChRs belong to the GPCR family and are therefore metabotropic receptors that are made up of 1 of 5 subunits: M1-5 (Chen et al., 2019). Depending on the G-protein that the subunit is coupled with, mAChRs will exert differing physiological functions. M1, M3 and M5 subunits belong to the Gq/G11 G-protein family and M2 and M4 subunits belong to the Gi/G0 G-protein family (Wess et al., 1997). Signalling pathways activated through M1, M3 and M5 receptors include phospholipases C, A2 and D, tyrosine kinase and calcium channels. Phospholipase A2 can also be activated through M2 and M4 receptors, which also show inhibition of adenylyl cyclase (Chen et al., 2019).

Structural visualisations of mAChRs have only recently been developed, starting with M2 (Kruse et al., 2012) and M3 (Haga et al., 2012) and all five subunits show a similar structure (Figure 1.11.): an extracellular ligand-binding site which is a pocket made up by 7 α-helix transmembrane domains, 3 of which are positioned at a perpendicular angle to the membrane and the other 4 are positioned at acute angles to the membrane (Baldwin et al., 1997; Maeda et al., 2019). G-proteins will interact with the loops at the intracellular domain (Halder & Lal, 2021).

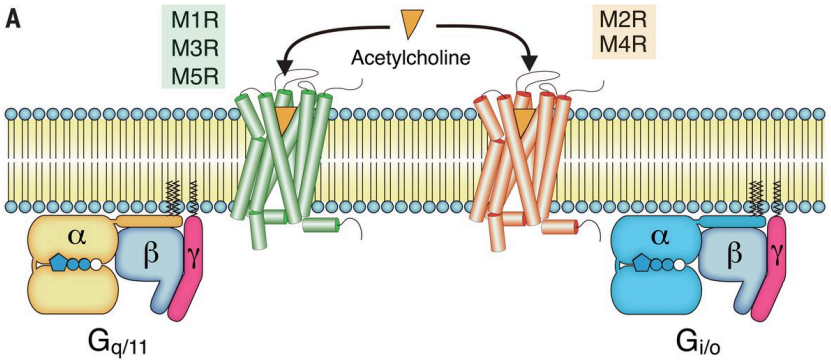


Figure 1.11. MAChR structure. M1-5 receptors have similar structures with the only difference being the G-protein that the receptor is coupled with. M1, M3 and M5 will couple with the Gq/11 protein and M2 and M4 will couple with the Gi/0 protein. Taken from Maeda et al. (2019).

MAChRs are found in a wide range of pre- and post-synaptic regions in the brain, including in the hippocampus, cerebral cortex, and the striatum. Activation of mAChR will be responsible for a variety of different functions including decision making (Goldberg et al., 2012), cognitive function (Park et al., 2019) and dopamine release (Zuccolo et al., 2019). mAChRs are also located in the heart where their activation can reduce contractions and action potential firing therefore lowering heart rate (Moss et al., 2018). Adverse effects due to mAChR activation are also seen in the gastrointestinal system, contributing to bowel obstruction due to tissue remodelling (Chen et al., 2020) and in the respiratory system, where overexpression of M3 receptors stopped the contraction of airways (Urso et al., 2020).

1.19. Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors, like $GABA_A$ receptors, also belong to the Cys-loop pentameric LGIC superfamily. nAChRs are made up of 5 subunits and the location of the receptor means that nAChRs can be classified as either neuronal, made up of 5 of the following 12 subunits: α2-10 and β2-4, or muscular, made up of the following 5 subunits: α1, β1, γ, δ and ε (Karlin, 1993). Subunits can either be assembled in a homo- or heteropentameric structure (Figure 1.12. A) with the pocket for ligand binding being formed where the subunits meet at "loops" (Figure 1.12. B) (Ho et al., 2020). Subunit structure is very similar to those in $GABA_A$ receptors, where loops are formed by an extracellular amino acid group on each

subunit, followed by 4 transmembrane domains, M1-4, with a large intracellular loop between M3 and M4 and an extracellular C-terminus (Figure 1.13.) (Gotti & Clementi, 2004). Subunits are arranged to form a central non-selective ion pore in the centre, which when opened after receptor activation, will allow the transport of cations such as Ca^{2+} (Beker et al., 2003), Na^{+} (Cohen et al., 1992) and K^{+} (Buisson et al., 1996). Cation transport into the cell results in depolarisation and the firing of action potentials, hence why nAChRs are considered excitatory receptors (Dani, 2015). Nicotine is known to have a high affinity for the $\alpha4\beta2$ subunits (Exley et al., 2011; McGranahan et al., 2011) however interactions of nicotine with other subunits has been demonstrated to have an effect: $\alpha3\beta4$ subunits have shown to induce bradycardia following nicotine consumption (Aberger et al., 2001) and $\alpha7$ may play a role in both memory (Levin et al., 1999) and sensory processing (Hajós et al., 2005) and may also be more permeable to Ca^{2+}, showing faster kinetics (Dani & De Biasi, 2001).

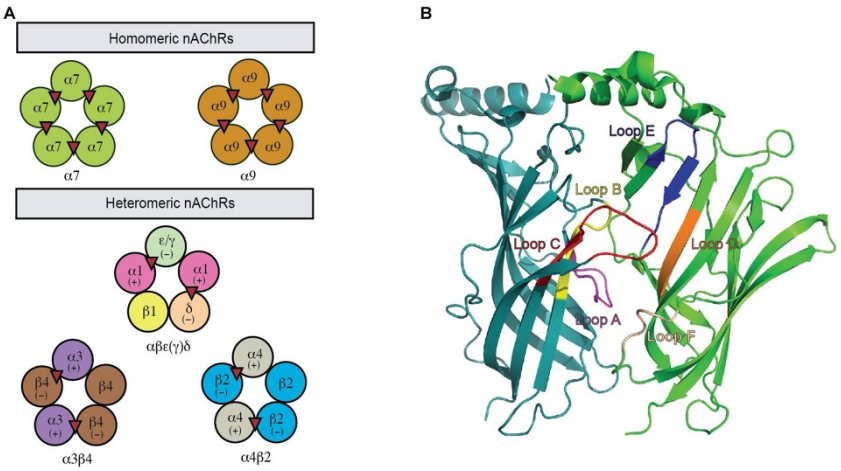


Figure 1.12. NAChR structure. (A) NAChRs can be either homopentameric or heteropentameric depending on the subunits. The red triangles represent the ligand-binding pockets which are enclosed where **(B)** the subunits meet to form "loops". Taken from Ho et al., (2020).

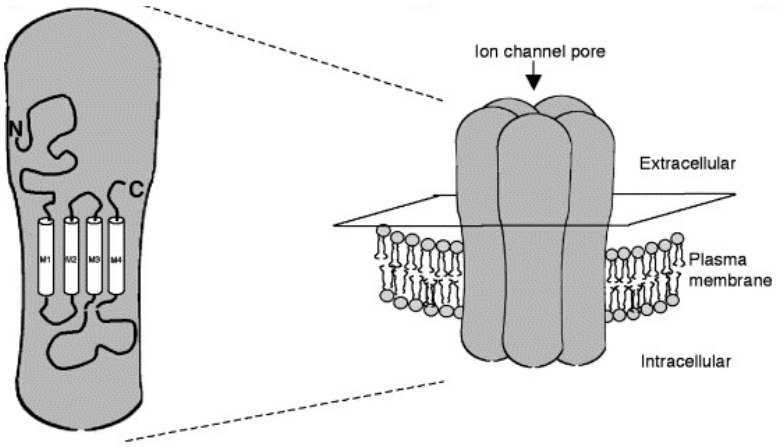


Figure 1.13. NAChR subunit structure. Within each subunit, there are 4 hydrophilic domains which will cross the membrane. The 5 subunits are arranged in a circular position, with the ion channel pore being formed in the middle. Taken from (Gotti & Clementi, 2004).

Within normal physiological states, nAChRs are responsible for a variety of different functions such as cognitive function including memory (Levin & Simon, 1998), neuronal development (Role & Berg, 1996) and the natural reward system (Robinson, 1993), which can also be utilised by addictive drugs.

Nicotine exerts its effect through the cholinergic system, acting as an exogenous agonist at nAChRs, modulating the mesolimbic dopaminergic system through which it exerts its rewarding properties (Zevin et al., 1998). Activation of the nAChR by nicotine-binding will open the ion channel, allowing an influx of Ca^{2+} and the firing of an action potential, before closing the ion channel. Mediated by $\alpha 4\beta 2$ nAChRs, this has been associated with the depolarisation of dopamine receptors causing an increase in the release of dopamine (Corrigall et al., 1994; di Chiara & Imperato, 1988) as well as other neurotransmitters such as acetylcholine (Summers et al., 1994) and serotonin (Bhalsinge et al., 2017). Nicotine-induced glutamate release contributes to these reinforcing properties as glutamate will activate dopamine and NMDA receptors, resulting in a long-term rewarding sensation (Mansvelder & McGehee, 2000). The spread of locations of nAChRs across preterminal and pre- and post-synaptic neurons mean that when nicotine enters the brain, it can activate the receptors at any of these locations (Dani et al., 2001). Desensitisation of the nAChRs to nicotine occurs when there is prolonged exposure to low concentrations of nicotine and this results in

tolerance to nicotine (Dani et al., 2000; Wooltorton et al., 2003). Whereas 1 mM acetylcholine is rapidly delivered to the synapse and readily broken down by acetylcholinesterase (AChE), 50-300 mM nicotine from tobacco will take longer to reach the synapses and as it is not broken down by AChE, will be present for longer (Dani & De Biasi, 2001; Gourlay & Benowitz, 1997). Brain imaging has shown that cigarette smokers show $\alpha4\beta2$ nAChR saturation throughout the day which was also shown in the same study to reduce cravings for nicotine (Brody et al., 2006); receptor saturation maintains the receptor desensitisation and allows the smoker to avoid any effects of nicotine withdrawal (Benowitz, 2009). In their desensitised form, nAChRs will show a higher affinity for nicotine than in their open and closed forms and can stay desensitised or non-functional for long periods of time (Lester & Dani, 1994; Margiotta et al., 1987). Desensitisation can also result in an increase in the number of nAChRs, known as upregulation, as the body attempts to maintain homeostasis following chronic nicotine administration and this is mediated by the $\alpha4\beta2$ nAChR (Buisson & Bertrand, 2001). Following removal of nicotine from the cholinergic system, the desensitised receptors will recover and become active again, resulting in an increase of excitatory nAChR activity responsible for the restlessness and agitation experienced during nicotine withdrawal and motivation for a smoker's next cigarette (Dani & De Biasi, 2001).

1.20. Mecamylamine

Mecamylamine is a non-competitive nAChR antagonist and was one of the first medicinal agents used to target nAChRs (Banerjee et al., 1990), originally introduced by Merck & Co to lower high blood pressure (Stone et al., 1956). Absorption of mecamylamine occurs in the gastrointestinal tract and easily crosses the blood-brain barrier to allow for its distribution in the CNS where it will bind with nAChRs (Suchocki et al., 1991). In *Xenopus* oocytes expressing both neuromuscular and neuronal nAChRs, mecamylamine inhibits all receptor activity (Chavez-Noriega et al., 1997) and in rat striatum, mecamylamine inhibits nicotine-induced dopamine release (Nickell et al., 2013). Mecamylamine does not stop nicotine binding to the nAChR (Banerjee et al., 1990); hypotheses of how mecamylamine acts as an antagonist involve the binding of mecamylamine within the nAChR ion channel where it is "trapped", preventing the transport of cations through the channel (Nickell et al., 2013; Ostroumov et al., 2008). Mecamylamine has been used in preclinical studies to observe the nAChR-mediated

rewarding effects of nicotine. In these studies, dose-dependent self-administration of nicotine has been shown to decrease following mecamylamine administration (Donny et al., 1999; Liu et al., 2007; Mansbach et al., 2000), demonstrating that nicotine-seeking and reinforcement of nicotine occurs through the nAChRs. Glutamatergic signals have also been shown to decrease following mecamylamine administration (Clarke et al., 1994). Interestingly, mecamylamine has also been shown to reduce ethanol consumption in rats and mice (Farook et al., 2009; Le et al., 2000).

1.21. Tubocurarine

Tubocurarine, a nondepolarizing neuromuscular blocking agent (NMBA) (Jonsson et al., 2006) derived from poisonous plants such as *C. tomentosum* and *S. toxifera*, is also an antagonist of nAChRs. However, unlike mecamylamine, tubocurarine is competitive for both orthosteric and allosteric binding sites on nAChRs, stopping ACh from binding (Brams et al., 2011; Ho et al., 2020). Tubocurarine can be administered orally, where it is will undergo absorption in the small intestine (Mahfouz, 1949) however clinically, administration is normally via injection intravenously where it will bypass absorption and metabolism and enter directly into the circulation where it will be distributed to the brain and excreted either renally or via salivary glands (Fisher et al., 1982; MA, 1949; Vardanyan & Hruby, 2006). Its role as a muscle relaxant meant it was first used as an anaesthetic in 1946 (Bowman, 2006). Similar to mecamylamine, tubocurarine has been demonstrated to block the nAChRs in *Xenopus* oocytes, where acetylcholine-induced depolarisation was inhibited following tubocurarine administration (Chavez-Noriega et al., 1997; Jonsson et al., 2006). Tubocurarine, by binding and dissociating with the receptor repetitively does not completely inhibit receptor activity but reduces acetylcholine-mediated neurotransmitter release as it reduces how often the channel is open (Bowman, 2006; Sheridan & Lester, 1977). This effect is readily reversible with the use of anticholinesterases (Aronson, 2016). Studies linking a reduced nicotine-induced toxicity due to tubocurarine have been done. Nicotine-induced cell proliferation has been shown to be dose-dependently reduced by tubocurarine administration in human bone cells (Walker et al., 2001) and vascular smooth muscle cells (Pestana et al., 2005). Tubocurarine administration has also been shown to inhibit the nicotine-induced cell apoptosis pathway (Hakki et al., 2001).

Both mecamylamine and tubocurarine are used as off-label treatments for smoking cessation (Crooks et al., 2014; National Center for Advancing Translational Sciences (NCATS), 2022). There is limited clinical data on the use of tubocurarine as a treatment to stop smoking. Clinical data for mecamylamine shows that whilst administering mecamylamine on its own can result in an increase of cigarette smoking (Rose et al., 1994a), a combination of mecamylamine and nicotine may be more beneficial in smoking cessation than nicotine alone (Lundahl et al., 2000; Rose et al., 1994a), as it can reduce cravings in heavy smokers, helping 50% of individuals quit smoking within 2 weeks of treatment (Jiloha, 2014).

1.22. Nicotine Toxicity

Nicotine-induced toxicity at a cellular level, like ethanol, can be due to the increased formation of ROS causing oxidative stress. Within rat pancreatic tissue, incubation in nicotine caused a significant increase in the rate of ROS formation, which was then inhibited by the administration of catalase suggesting that the ROS responsible for nicotine toxicity are superoxide anions and hydroxyl, produced from hydrogen peroxide (Wetscher et al., 1995). In hamster cells, a similar increase in the production of ROS was observed following isolated nicotine administration (Yildiz et al., 1999). Husain et al. (2001) demonstrated that there is a nicotine-induced reduction in glutathione (GSH) levels in liver and testicular cells of rats that have been administered nicotine subcutaneously. As GSH is a detoxifying scavenger for ROS (DeLeve & Kaplowitz, 1991), its reduced levels will lower the rate of ROS removal and therefore contribute to oxidative stress. Lipid peroxidation due to this oxidative stress was also demonstrated in the same study when increased lactate dehydrogenase (LDH) activity was seen in the media, suggesting that the cell membranes had been disrupted and LDH had leaked out (Yildiz et al., 1999). Lipid peroxidation, due to ROS (as described in 1.2.4.) is responsible for the toxic effects seen with isolated nicotine consumption such as cardiovascular damage such as atherosclerosis, respiratory damage, such as COPD and endothelial dysfunction (Ambrose & Barua, 2004; Frei et al., 1991), all of which are exacerbated when nicotine is smoked as ROS have also been shown to form within a burning cigarette in both the cigarette smoke in gaseous form, where they will interact with the

respiratory tract, and in the butt of the cigarette as particulate matter, where they can contribute to the production of more ROS (Huang et al., 2005).

The HSP system has been suggested to be activated by the oxidative stress due to nicotine, therefore suggesting the role of HSPs as biomarkers for nicotine toxicity. Whilst an older study by Hahn et al. (1991) in hamster cells demonstrated that upregulation of HSP70 was only seen with nicotine when heat was applied, more recent *in vitro* studies have demonstrated that nicotine has been shown to upregulate HSP70 in rat motoneurons (Corsini et al., 2017) and kidneys (Wedn et al., 2019). A preliminary *in vitro* report has also demonstrated an increase in macrophagic HSP60 seen following administration of electronic cigarette smoke with and without nicotine, with nicotine-containing smoke causing a significantly higher increase in HSP60 (Rahman et al., 2022). HSP90 has also been shown to be produced in rats' livers following nicotine administration (Bagchi et al., 1995) and involved in nicotine-induced human cell apoptosis (Wu et al., 2002). An *in vivo* study carried out in *C. elegans* showed that a low nicotine administration demonstrates an increase in the presence of HSP60 by 7.5-fold than the control (Sobkowiak et al., 2017). Cigarette smokers show increased levels of HSP70 compared to non-smokers in blood (Moreira Santos et al., 2017) and saliva (Bobbili et al., 2020) and similarly, cigarette smoke has been shown to active the HSP60 pathway in endothelial cells, where it is responsible for the pathogenesis of atherosclerosis (Kreutmayer et al., 2011) and mononuclear blood cells, where it is responsible for the pathogenesis of chronic pulmonary obstructive disorder (COPD) (Ou et al., 2022) however it is not confirmed whether these are due to nicotine exposure or other chemicals present in the smoke.

Malondialdehyde (MDA) can be used as a biomarker for lipid peroxidation and smokers have been shown to have increased levels of MDA compared to non-smokers, suggesting its role in nicotine toxicity (Kamceva et al., 2016). As it is produced by an increase in ROS, it can signal if there is any tissue damage (Gaweł et al., 2004). Yildiz et al. (1999) used MDA to demonstrate the increase in ROS formation in hamster cells and more recently, following isolated nicotine administration, Khademi et al. (2019) used an increase in the levels of MDA in human endometrial cells to demonstrate the toxicity of direct nicotine administration in the endometrium.

Nicotinic metabolites can also be used as biomarkers for nicotine intake and toxicity. Cotinine has a longer half-life than nicotine and can therefore be detected in blood plasma, urine and saliva for a longer period of time to confirm nicotine consumption (Benowitz et al., 2009). Evidence for the role of cotinine's precursor, nicotine-Δ1' (5')-iminium ion, in electron transfer, ROS formation and oxidative stress means that the iminium ion can be used as a biomarker for nicotine toxicity (Kovacic & Cooksy, 2005).

1.23. The use of *Lumbriculus variegatus* as an alternative *in vivo* model

Lumbriculus variegatus (*L. variegatus*), known also as the California blackworm, is an aquatic oligochaete worm part of the Annelida phylum. It is found globally in freshwater lakes and marshes, in temperatures ranging from 4 – 15°C (Daoud et al., 2022). Within its ecosystem, *L. variegatus* carries out sedimentary organic material decomposition and is a primary consumer within the food webs in freshwater environments, acting as food for animals higher in trophic levels (Daoud et al., 2022; O'Gara et al., 2004; Williams, 2005).

When observing the structure of *L. variegatus*, there is often a colour gradient from the darker anterior end containing the conical prostomium (the head), brain, mouth, digestive system, and hermaphroditic sexual organs, to the lighter posterior end containing the respiratory functions and photoreceptor cells (Figure 1.14.) (Alkhathlan, 2015).

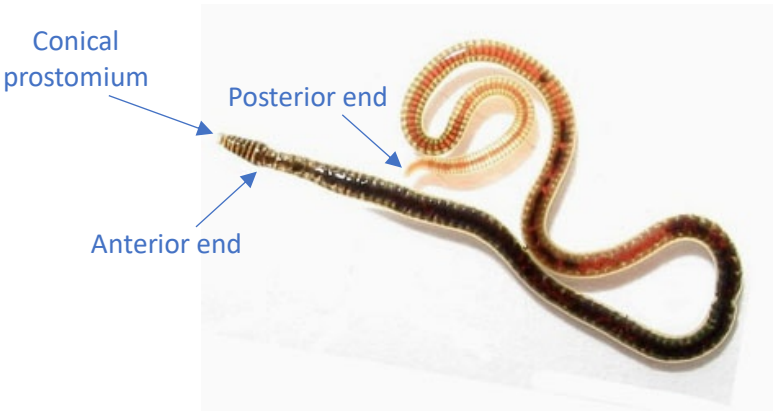


Figure 1.14. Labelled image of *L. variegatus*. The anterior and posterior ends and conical prostomium have been labelled. Image taken from https://www.biologycorner.com/2021/08/14/investigation-how-chemicals-impact-pulse-rates/#google_vignette.

Whilst the digestive system has not yet been described for *L. variegatus*, the worms will submerge their head into the sediment to find food, with their diet consisting of algae, decaying plants and bacteria (Williams, 2005). Any undigested material is passed at sediment surface level in the form of faecal pellets. This is known as the "conveyor-belt" feeding system (Gebhardt & Forster, 2018) as the faecal pellets produced by *L. variegatus* alter the top layer of sediment biologically, chemically, and physically (Williams, 2005).

When the head of the worm is burrowed in the sediment, the tail will be left out in the water for gas exchange to occur. As the posterior end of the worm has lower body muscle, it is well adapted to allow gas exchange via cutaneous respiration to occur at the dorsal surface (Drewes, 1990; Halfmann & Crisp, 2011).

Oligochaete worms have a closed circulatory system made up of two major blood vessels: dorsal blood vessel (DBV) and ventral blood vessel (VBV) (Figure 1.15.). A pair of smaller vessels, lateral commissural vessels, is present in each of the anterior segments in the worms, connecting the two major vessels with these segments. Branched lateral vessels extend from the DBV in most segments. These vessels contract in unison with the DBV and rather than connect with the VBV, they end "blindly". Whilst *L. variegatus* contain a bright red blood, the haemoglobin-like respiratory pigment erythrocruorin is not absorbed in red blood cells as in vertebrates but is absorbed in the plasma (Lesiuk & Drewes, 1999b). Blood flow through the worm begins at the posterior end of the DBV where oxygenated blood is pumped through the worm via waves of muscle contractions. More than one wave can be observed at a time along the worm's body however the rate at which they occur will vary. Lesiuk & Drewes, (1999) illustrated that the pulse rate at the posterior end ranged from 24 to 32 beats per minute (bpm) compared to 8 to 12 bpm at the anterior end. This is suggestive of the pulse wave not travelling across the full length of the DBV. A more recent study by Crisp et al., (2010) demonstrated that the DBV pulse rate of *L. variegatus* is regulated by endogenous biogenic amines, specifically serotonin and dopamine.

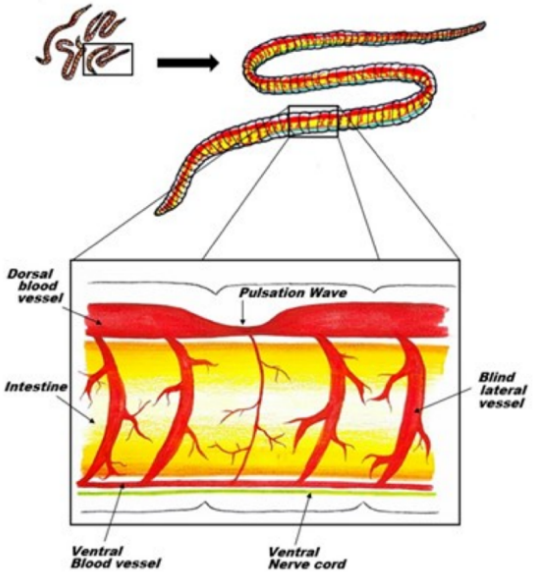


Figure 1.15. Cross section of the *L. variegatus* closed circulatory system. The two main blood vessels, the dorsal blood vessel (which is larger) and the ventral blood vessel and the smaller blind lateral vessels are labelled. The pulsation wave is caused by the muscle contractions and is used to measure the bpm of the worm (taken from Ryan & Elwess (2017)).

Certain *L. variegatus* characteristics and processes can differ in their natural habitat compared to when kept in the laboratory. In their natural habitat, adult *L. variegatus* can grow to around 10 cm in length (Alkhathlan, 2015) however within the laboratory, their growth is normally shortened to around 5 – 8 cm in length (Seeley et al., 2021). Reproduction of *L. variegatus* also differs between their natural environment and within the laboratory: naturally, *L. variegatus* will sexually reproduce and embryos are held by transparent cocoons that are laid by the worm, whereas within the laboratory, sexual maturity does not occur and therefore the worms will asexually reproduce via a process known as asexual fragmentation (Alkhathlan, 2015). An autotomy reflex will result in a sudden circular muscle contraction at a specific site on the segment which results in the worm splitting into two or more fragments. These fragments will then undergo regeneration to form new body segments which will then grow into a new worm (Alkhathlan, 2015; Martinez Acosta et al., 2021). An epidermal serotonin immunoreactive nerve ring will identify the site at which fragmentation will occur (Martinez, 2006) The exact mechanism by which the autotomy reflex is activated is still unknown, however nicotine, a cholinergic agonist, has been demonstrated to inhibit the reflex; this

evidence suggests that the activation of the reflex involves the activation of serotonergic neurons via the cholinergic system (Lesiuk & Drewes, 1999a, 2001).

Regeneration in response to injury will differ to the regeneration that occurs during asexual fragmentation. It can occur via morphallaxis, when present tissue is remodelled, or via epimorphosis, when current tissue undergoes dedifferentiation, forming an undifferentiated group of cells which will then redifferentiate into new tissue (Richmond, 2020).

The central nervous system (CNS) within *L. variegatus* is made up of a cerebral ganglion in the conical prostomium and a ventral nerve cord which stretches through the full length of the worm (Lesiuk, 2000). A neuropil at the centre of the ventral nerve cord is where the sensory, motor and interneurons all meet. These connections are responsible for *L. variegatus* behaviour and movement as they make up the neural circuits and reflex systems (Drewes, 2002). As a survival mechanism when their tail is exposed, a rapid withdrawal response, induced by tactile stimulation and mediated by the ventral nerve cord which is comprised of giant fibres, has developed to protect the tail. Medial giant fibres (MGF) are activated when the anterior end of the worm's body is stimulated, and lateral giant fibres (LGF) are activated when the posterior end of the worm's body is stimulated (Drewes, 2002). Activation of the giant fibres will lead to activation of the motor neurons which will in turn lead to activation of the longitudinal body wall muscles which will contract and cause *L. variegatus* to shorten its body. *L. variegatus* exhibits three quantifiable behaviours in regard to movement: body reversal, helical swimming, and free locomotion (O'Gara et al., 2004). Tactile stimulation of the anterior end (the head) results in body reversal and tactile stimulation of the posterior end (the tail) results in a corkscrew movement known as helical swimming (Drewes, 1999; O'Gara et al., 2004).

L. variegatus show sensitivity to varying environmental stimuli such as light, slight pressure (triggered by touch) and oxygen deficiency (Daoud et al., 2022). Size and movement of *L. variegatus* allow for ease in visibility and due to being an invertebrate model, they are not covered by ASPA and contribute towards the efforts of the NC3Rs. The above characteristics make *L. variegatus* a suitable invertebrate model for many areas in scientific research.

Ecotoxicology studies have widely utilised *L. variegatus* and its behavioural responses as an endpoint model to observe sedimentary toxicology and bioaccumulation due to their habitat and feeding habits (Phipps et al., 1993). O'Gara et al., (2004) used *L. variegatus* to demonstrate the sublethal effects of copper entering aquatic environments. More recently, bioassays completed by Wallin et al. (2018) expanded on this, using *L. variegatus* to demonstrate not only copper toxicity in aquatic environments but also toxicity caused by other mining by-products like sulphur, nickel, and uranium.

More recently, *L. variegatus* has been utilised in pharmacology and toxicology studies to expand its use beyond ecotoxicology and explore its physiological and behavioural responses to drug compounds. Examples include antidepressant fluoxetine, a selective serotonin reuptake inhibitor, that was shown to significantly increase reproduction rates of *L. variegatus* (Nentwig, 2007) and antibiotic triclosan, also an anti-fungal agent, increasing *L. variegatus* feeding (Karlsson et al., 2016). Another antidepressant, clozapine (antagonist of various serotonin receptors) was used when demonstrating the role of biogenic amines in DBV pulse rates (Crisp et al., 2010). *L. variegatus* have demonstrated significant decreases in movement in response to increasing concentrations of lidocaine, a sodium channel blocker and quinine, a sodium and potassium channel blocker (Seeley et al., 2021).

Bellamy (2023) exposed *L. variegatus* to a pharmacologically diverse range of compounds including ion channel blockers, such as lidocaine and quinine, as well as neurotransmitters such as dopamine and GABA, the $GABA_A$ receptor antagonist bicuculline and ethanol. Following 10-minutes exposure to $\geq$250 mM ethanol, a significant decrease in the stereotypical movements, body reversal and helical swimming, and free locomotory movement was observed (Appendix Figure 1 A-B & F). These effects were then reversed, and movement returned to baseline for all ethanol concentrations 10-minutes and 24 hours following ethanol removal (Appendix Figure 1 C-D & G). These results established that the lowest observable adverse effect level (LOAEL) for ethanol was 250 mM and the no observable adverse effect level (NOAEL) for ethanol was 100 mM. Within the Swansea Worm Integrative Research Laboratory, administration of $\geq$0.1 mM nicotine also induced a significant decrease in the stereotypical movements, body reversal and helical swimming, and free locomotory movement (Appendix Figure 7 A-B & F) (Carriere, 2022). Unlike ethanol however, these effects

were not reversed 10 minutes following nicotine removal but were reversed 24 hours following nicotine removal, except for *L. variegatus* exposed to 0.25 mM nicotine, who still exhibited reduced free locomotory movement following 24 hours after nicotine removal (Appendix Figure 7 C-D & G) (Carriere, 2022). Data gathered by Bellamy (2023) and the Swansea Worm Integrative Research Laboratory was used as a foundation to further elucidate *L. variegatus* as a novel *in vivo* model during this study.

1.24. Aims and objectives

Pre-clinical *in vivo* studies observing the effects of drugs of abuse will commonly use rodent models such as rats and mice (Spanagel, 2017). Championed by framework such as ASPA within the UK and the 3Rs, there is a growing need to develop alternative *in vivo* models.

During this project, the use of the novel aquatic oligochaete *L. variegatus* as an *in vivo* model for the drugs of abuse, ethanol and nicotine, will be observed. This project aims to:

- Establish a pharmacological profile of acute and chronic ethanol administration to *L. variegatus* by:
    - Utilising the optimised *in vivo* behavioural assays to observe *L. variegatus* behavioural response to ethanol with and without pre-treatment of GABA receptor agonists and antagonists.
    - Utilising the behavioural assays to observe *L. variegatus* behavioural response to chronic ethanol administration compared to acute ethanol administration.
    - Utilising *in vitro* assays to establish the toxicity of acute and chronic ethanol treatment in *L. variegatus.*
- Establish a pharmacological profile of acute nicotine administration by:
    - Utilising the optimised *in vivo* behavioural assays to observe *L. variegatus* behavioural response to nicotine with and without pre-treatment or co-administration of nAChR antagonists.
    - Utilising *in vitro* assays to establish the toxicity of acute nicotine treatment in *L. variegatus.*

2. Materials and Methods

2.1. Safety

Manufacturer's instructions were followed regarding disposal of all waste. Analysis of all experimental procedures was completed and control of substances hazardous to health (COSHH) forms were used to conduct formal and register formal risk assessments.

2.2. Reagents and solution

Table 2.1. Reagents inventory. Below is a list of reagents, where they were supplied from and how they were stored within the laboratory.

Reagent	Supplier	Storage
Baclofen	MedChem Express	2-4°C
Bicuculline	Sigma-Aldrich	Room temperature
Bovine serum albumin (BSA)	ThermoFisher Scientific	2-4°C
Bradford assay	ThermoFisher Scientific	2-4°C
Bromophenol blue	Sigma-Aldrich	Room temperature
Calcium nitrate tetrahydrate	Duchefa Biochemie	Room temperature
Chloroform	ThermoFisher Scientific	Room temperature
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Room temperature
Dithiothreitol	Melford Laboratories	2-4°C
Ethanol	Fisher Chemical	Room temperature
GABA	Sigma-Aldrich	Room temperature
Glycerol	Melford Laboratories	Room temperature
Glycine	Sigma-Aldrich	Room temperature
HEPES	Melford Laboratories	Room temperature
Magnesium sulphate heptahydrate	Duchefa Biochemie	Room temperature
Mecamylamine	CalBioChem	2-4°C
Methanol	Fisher Chemical	Room temperature
Marvel Skimmed Milk powder	Marvel	Room temperature
Nicotine	Sigma-Aldrich	Room temperature
NP-40	Sigma-Aldrich	Room temperature
Protease inhibitor cocktail (PIC)	Calbiochem® 539134	-20°C
Potassium chloride	Melford Laboratories	Room temperature
Sodium deoxycholate	Sigma-Aldrich	Room temperature
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Room temperature
Sodium chloride	Melford Laboratories	Room temperature
Sodium hydroxide	Sigma-Aldrich	Room temperature
Tris, Hydrochloride	Melford Laboratories	Room temperature
Tubocurarine	Tocris	2-4°C

2.3. Storage and preparation of drugs and solutions

Artificial pondwater (APW)

1 mM sodium chloride, 13 μM potassium chloride, 4 μM calcium nitrate tetrahydrate, 17 μM magnesium sulfate heptahydrate and 71 μM HEPES pH buffer was dissolved in up to 1 litre of deionised water to make 100x APW (as per O'Gara et al., 2004). 100x APW was diluted to make 1x APW.

Baclofen

Artificial pondwater at 65°C, with sonification, was used to dissolve baclofen to make a 20 mM stock concentration. Dilution of this stock into working concentrations of 1 – 20 mM was done using artificial pond water.

Bicuculline

100% DMSO at 50°C was used to dissolve bicuculline to make a 500 mM stock concentration. This stock was divided into 200 μL aliquots and stored at -80°C. When ready to use, an aliquot(s) was thawed and dilution of this stock into a working concentration of 2.5 mM was done using artificial pond water.

Enhanced luminol-based chemiluminescent

Clarity enhanced luminol-based chemiluminescent (ECL) was purchased from Bio-Rad and made up in a 1:1 solution following manufacturer's guidelines.

Ethanol

Artificial pondwater was used to dilute 99% ethanol to make working concentrations of 2.5 – 500 mM.

GABA

Artificial pondwater was used to dissolve GABA to make a 100 mM stock concentration. Dilution of this stock into working concentrations of 0.1 – 100 mM was done using artificial pondwater.

Mecamylamine

Artificial pondwater was used to dissolve mecamylamine to make a 100 μM stock concentration. Dilution of this stock into working concentrations of 0.1 – 100 μM was done using artificial pondwater.

Nicotine

Artificial pond water was used to dilute nicotine to make a working concentration of 1 mM. When completing combination experiments, nicotine was diluted using the antagonist solution to produce 0.1 mM nicotine.

Radioimmunoprecipitation assay (RIPA)

10 mL of RIPA lysis buffer was made up using 0.3 mL of 5M sodium chloride solution, 1 mL of 10% NP-40 solution, 0.5 mL of 10% sodium deoxycholate solution, 0.5 mL of 1M Tris pH 8.0 and 0.1 mL of 10% sodium dodecyl sulfate and adding up to 10 mL of deionised H_2O. This was divided into aliquots of 500 μL and stored at -20^{o}C.

10X Running Buffer

10X Novex™ Tris-Glycine SDS running buffer was purchased from ThermoFisher and diluted to 1X running buffer following manufacturer's guidelines.

Sample buffer

6x sample buffer was made up using 350 mM Tris pH 6.8, 30% glycerol, 10% SDS, 600 mM dithiothreitol and 62.5 mg powdered 0.12% bromophenol blue.

2x sample buffer was made by diluting 6x sample buffer with sterile ddH2O in a 1:3 ratio

20X TBS-T

1L 20X TBS-T solution was made up using 600 mL of 5 M sodium chloride solution, 200 mL 1M Tris buffer pH 8.0 solution and 10 g of Tween-20.

25X Transfer Buffer

25X Tris-Glycine transfer buffer was purchased from ThermoFisher and diluted to 1X transfer following manufacturer's guidelines.

Tubocurarine

Artificial pondwater was used to dissolve tubocurarine to make a 100 μM stock concentration. Dilution of this stock into working concentrations of 1 – 100 μM was done using artificial pondwater.

2.4. Culturing *L. variegatus*

Cultures of *L. variegatus* were obtained from ALFA Fish Food and were reared in the laboratory in aquariums with artificial pond water, as is previously described (O'Gara et al., 2004a; Seeley et al., 2021). Artificial pond water was composed of the following: 1 mM sodium chloride, 13 μM potassium chloride, 4 μM calcium nitrate tetrahydrate, 17 μM magnesium sulfate heptahydrate, 71 μM HEPES buffer. *L. variegatus* were kept in a 16:8-hour light-dark cycle at room temperature. There was continuous filtration and aeration (using air stones) of artificial pond water in the aquaria. TeraMin flakes and 10 mg/L spirulina were used weekly to feed the worms. Following 3 months of culture maintenance, experimentation could begin. The aquaria were maintained weekly. 24 hours prior to experimentation, individual worms were randomly selected, ensuring that they did not have any obvious structural irregularities, and placed into 6-well plates (Cellstar®).

2.5. Ethanol culture

A population of *L. variegatus* exposed to ethanol was also grown in a separate aquarium. Artificial pond water was made up as above with the addition of ethanol to make a final concentration of 100 mM ethanol, which is equivalent to 0.46% and therefore higher than human exposure. *L. variegatus* were subjected to the same light-dark cycles, temperature and feeding routine as the ethanol-naïve cultures. The ethanol culture aquarium was cleaned twice weekly. Following 21 days of culture maintenance, experimentation could begin. Experimental design was conducted as above with individual worms randomly selected 24 hours before experimentation.

2.6. Stereotypical movement assay

Individual worms were placed in a 6-well plate (Cellstar®), with one worm per well, 24 hours prior to experimentation. To record the pre-drug exposure (Baseline) stereotypical movements of each worm, each well was washed and replaced with 4 mL clean artificial pondwater. Stimulation of the anterior and posterior ends result in body reversal and helical swimming movements respectively. A clean 20-200 μL pipette tip was used to stimulate each end of the worm (Figure 2.1. B & C), with a 5-10 second interval between stimuli. The ability

of each *L. variegatus* to perform body reversal and helical swimming was recorded on a scoring sheet (Figure 2.1. D) using the following scoring: 1 = No Movement, 2 = Partial Stereotypical Movement, 3 = Full Stereotypical Movement. The artificial pondwater was then removed from each well. To each well, either a vehicle (artificial pondwater) or the drug solution was added for 10 minutes (Drug Exposure). Tactile stimulation was then carried out again and scored. Each well was then washed to remove any trace of the drug solutions and replaced with clean artificial pondwater. *L. variegatus* were stimulated 10 minutes (Rescue 10 minutes) and 24 hours (Rescue 24 hours) following removal of the drug or vehicle (Figure 2.1.). Treatment times were not staggered per well.

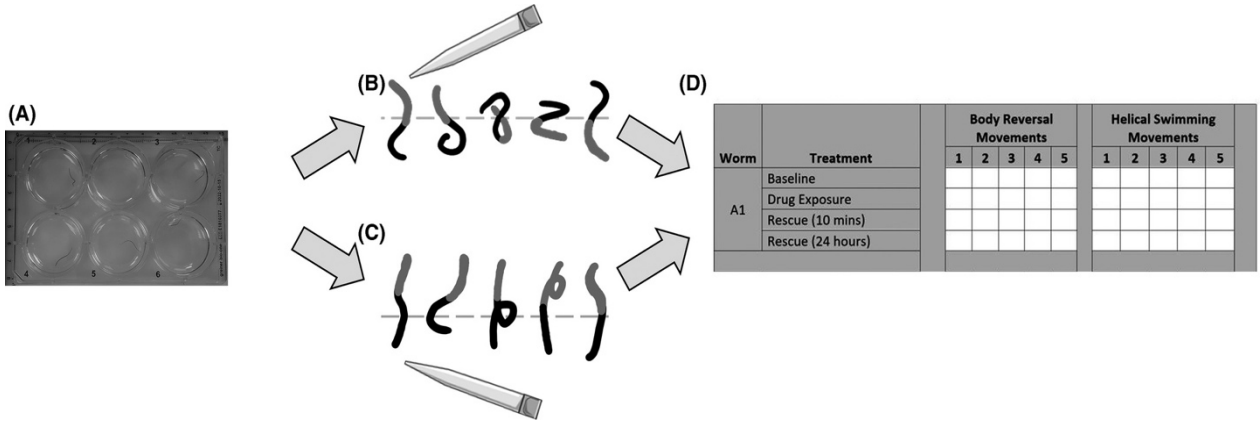


Figure 2.1. Assessing *L. variegatus* stereotypical movement. (A) 24 hours prior to experimentation, individual *L. variegatus* are placed in a 6-well plate (Cellstar®). A 20 – 200 µL pipette tip was then used to stimulate the **(B)** anterior and **(C)** posterior end of each *L. variegatus* for a total of 5 times on each end. **(D)** Scoring of the ability to perform stereotypical movement was rated as previously described by Seeley et al., (2021): 1 = No Movement, 2 = Partial Movement, 3 = Full Stereotypical Movement. Steps B-D were repeated per plate when observing stereotypical movement pre-drug exposure (Baseline), during 10-minute drug incubation (Drug Exposure), 10 minutes following drug removal (Rescue (10 mins)) and 24 hours following drug removal (Rescue (24 h)). Data for each stage is presented as a percentage ratio of movement when compared to Baseline movement (taken from Seeley et al., (2021)).

2.7. Free locomotion assay

Individual worms were placed in a 6-well plate (Cellstar®), with one worm per well, 24 hours prior to experimentation. To record the pre-drug exposure (Baseline) free locomotory movements of each worm, each well was washed and replaced with 2 mL clean artificial pondwater (this is to reduce movement in the z-axis). A 13-megapixel camera was used to collect rapid images, 1 image per second for 50 second. The artificial pondwater was then removed from each well. To each well, either a vehicle (artificial pondwater) or the drug solution was added for 10 minutes (Drug Exposure). Rapid image collection was then completed again. Each well was then washed to remove any trace of the drug solutions and replaced with clean artificial pondwater. *L. variegatus* were imaged 10 minutes (Rescue 10 minutes) and 24 hours (Rescue 24 hours) following removal of the drug or vehicle. ImageJ® was used to analyse the collected images. The 50 images per condition were superimposed to create a z-stack image. Measuring the known distance covered by each worm allowed the total area that *L. variegatus* moved during Baseline, Drug Exposure, Rescue (10 minutes) and Rescue (24 hours) to be calculated (Figure 2.2.).

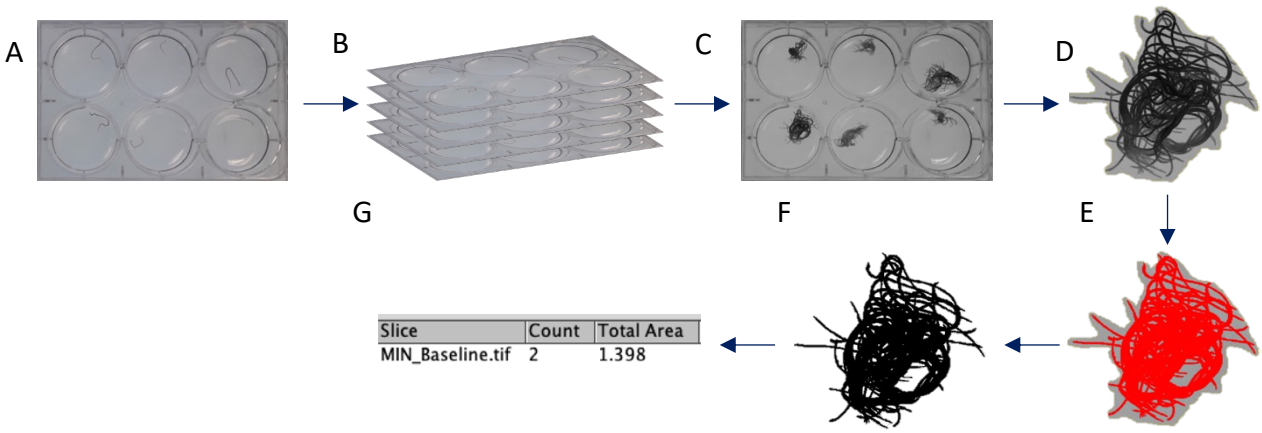


Figure 2.2. Assessing *L. variegatus* free locomotory movement. (A) 24 hours prior to experimentation, individual *L. variegatus* are placed in a 6-well plate (Cellstar®). **(B)** Images are rapidly collected with 1 image per second for 50 seconds and **(C)** the 50 images are superimposed into a z-stack. Using a known distance, a scale is set on the z-stack. **(D)** Freehand selection is used to outline each *L. variegatus*, removing the rest of the plate. **(E)** The image threshold is set to highlight only the worm followed by **(F)** eliminating the background to ensure only the worm outline can be seen. **(G)** The previously set scale is then used to calculate the total area travelled by each worm. Steps B-D were repeated per plate when observing stereotypical movement pre-drug exposure (Baseline), during 10-minute drug incubation (Drug Exposure), 10 minutes following drug removal (Rescue (10 mins)) and 24 hours following drug removal (Rescue (24 h)). Data for each stage is presented as a percentage ratio of movement when compared to Baseline movement (this has been adapted from Seeley et al., (2021)).

2.8. Onset of Action

Individual worms were placed in a 6-well plate (Cellstar®), with one worm per well, 24 hours prior to experimentation. To record the pre-ethanol exposure (Baseline) free locomotory movements of each worm, each well was washed and replaced with 2 mL clean artificial pondwater (this is to reduce movement in the z-axis). Images were rapidly collected, 1 image per second for 50 second. The artificial pondwater was then removed from each well. To each well, either a vehicle (artificial pondwater) or 25 – 500 mM ethanol solution was added, and rapid images were collected every 2 minutes for 10 minutes. Images were analysed as previously described in 2.6. (Figure 2.2.), using Image J®. Concentrations of ethanol used were higher than the relevant concentrations of human exposure (Lee et al., 2009).

2.9. Acute functional tolerance

Individual worms were placed in a 6-well plate (Cellstar®), with one worm per well, 24 hours prior to experimentation. Acute functional tolerance was observed utilising the free locomotion assay. To record the pre-drug exposure (Baseline) free locomotory movements of each worm, each well was washed and replaced with 2 mL clean artificial pondwater and the plate was imaged. The artificial pondwater was then removed from each well and 500 mM ethanol was administered to each well. *L. variegatus* were imaged following 10 minutes of exposure and then imaged at 20-minute intervals for a total of 210 minutes of continuous ethanol exposure. Images were analysed using ImageJ® as previously described.

2.10. Determining LD_{50} following chronic exposure

Individual worms were placed in a 6-well plate (Cellstar®), with one worm per well. Each well was washed and either the vehicle (artificial pondwater) or 25 – 500 mM ethanol solution was added. Ethanol solutions were made up using conditioned pond water from the aquarium in place of fresh pond water to ensure *L. variegatus* had access to nutrients due to the extended period outside of the aquarium. Ethanol solutions in the wells were changed daily. Every 24 hours, for 72 hours, the number of worms still alive were counted and recorded. Decomposition of body tissue, characterised by discoloration of the body, indicated the death of a worm.

2.11. Imaging size and movement

Individual worms from the ethanol naïve and ethanol cultures were placed in separate 6-well plates (Cellstar®) respectively, with one worm per well, 24 hours prior to experimentation. Pre-imaging, each well for both plates was washed and replaced with 2 mL clean artificial pondwater. Using a 13 megapixel camera, an individual image of each plate was taken. Single image analysis was completed as previously described in 2.6. (Figure 2.2.), using Image J® (due to the single image, a z-stack is not needed).

Worms collected for size analysis were re-used to analyse the movement of *L. variegatus*. Free locomotory images were captured and analysed as previously described in 2.6. (Figure 2.2.), using Image J®. Using equation 2.1., where z = area covered by worm, r = radius of the well and s = size of worm, inputting data from the size and movement analysis, movement of individual *L. variegatus* compared to size was calculated.

$$Relative\ movement = \frac{\left(\frac{z}{\pi r^2}\right) x\ 100}{s}$$

Equation 2.1. Equation calculating the relative movement of *L. variegatus* to body size. z = area covered by worm, r = radius of the well and s = size of worm.

2.12. Protein extraction

Individual worms were placed in a 6-well plate (Cellstar®), with one worm per well, 24 hours prior to extraction. During the protocol, all samples and reagents were kept on ice. Worms were moved to 1.5 mL centrifuge tubes, with 3 worms per centrifuge tube. After the removal of all pond water, 200 μL of fresh ice-cold pond water was used to wash the worms. After the removal of all pond water, into each centrifuge tube, 20 μL of fresh ice-cold pond water was added. Using the tissue homogeniser (Argos Technologies), each sample was homogenised, with the tip changed between each sample to avoid sample contamination. Snap-freezing of the samples at -80 °C was completed for 60 minutes.

The protease inhibitor cocktail (PIC) (Calbiochem®) and a 500 μL aliquot of Radioimmunoprecipitation assay (RIPA) were thawed on ice. PIC was added into the RIPA at a 1:1,000 dilution.

Once the snap frozen samples were thawed, 80 μL of RIPA + PIC was added to each sample which were left on ice for 30 minutes to lyse. In a pre-cooled centrifuge at 4°C, the lysed samples were spun at 16,100 x g for 15 minutes. The supernatant was extracted into fresh centrifuge tubes on ice and the pellet discarded.

2.13. Protein quantification

A standard curve was generated via the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) to quantify known protein concentrations. A 2 mg/mL BSA solution was diluted to make the following concentrations: 0, 1, 2, 4, 8, and 10 ug/mL. The absorbance on the spectrophotometer was set to a single wavelength of 595 nm. Protein absorbances were measured for each concentration, generating the following equation:

$$y = mx + c$$

Per cuvette, 1 μL of *L. variegatus* protein supernatant was dissolved in 1 mL of Bradford reagent. A cuvette containing 1 mL of Bradford reagent with 1 μL RIPA + PIC was used to blank the spectrophotometer. Protein absorbances for each sample were then measured and input into a spreadsheet which used the above equation to quantify the concentration of protein per sample. The volume of each sample containing 30 ug of protein was then calculated and transferred into clean centrifuge tubes.

To ensure each sample contained the same volume of protein, calculated volumes of RIPA buffer and 2x sample buffer (117 mM Tris pH 6.8, 10% glycerol, 3.3% Sodium Dodecyl Sulfate, 200 mM DithioThreitol and 0.04% bromophenol blue) were added to each protein sample, ensuring that the final loading volume did not exceed 40 μL. Samples were heated to 95°C on a pre-heated heating block, placed on ice and then stored at -20°C until required for experimentation.

2.14. SDS-PAGE, protein transfer, Western Blotting and densitometry analysis

2.14.1. SDS-PAGE

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins. As per manufacturer's guidelines, an Invitrogen Mini Gel Tank was used with the Novex® Tris-glycine SDS running buffer (Invitrogen).

500 mL of 1X running buffer was prepared for each tank. Each sample (with 30 ug of protein) was loaded onto 15-well Novex® Tris-glycine polyacrylamide gels, as per the loading volume calculated during protein quantification. Set at a running voltage of 100 V, SDS-PAGE was run until the proteins reached the bottom of the gel. The Page Ruler Plus Protein Ladder (ThermoFisher Scientific) was also loaded onto the gel to provide a reference for the protein size (Figure 2.3.).

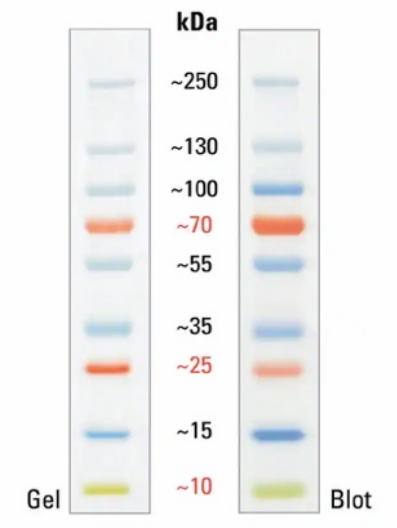


Figure 2.3. PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa. During protein separation, the protein ladder was used to indicate the sizes of protein on the gel (taken from ThermoFisher Scientific).

2.14.2. Protein transfer

Proteins on the gel were transferred on to a membrane. As per manufacturer's guidelines, an Invitrogen Mini Gel Tank was used with the Tris-Glycine Transfer Buffer (ThermoFisher).

For each tank, 500 mL of 20% methanol 1X transfer buffer was prepared; this solution was used to soak two sponges (ensuring all air bubbles were removed) and two pieces of filter paper. A piece of polyvinylidene difluoride (PVDF) membrane, cut to be the same size as the gel, was activated in methanol. Placing the Mini Blot Module cathode (-) core on a flat surface, the module sandwich was assembled (Figure 2.4.). Any air bubbles were removed with a blotting roller and the membrane was handled using a pair of tweezers, to avoid any protein contamination. Ensuring that the module was fully saturated in the transfer buffer, protein transfer was completed at 20V (for the PVDF membrane) for 60 minutes.

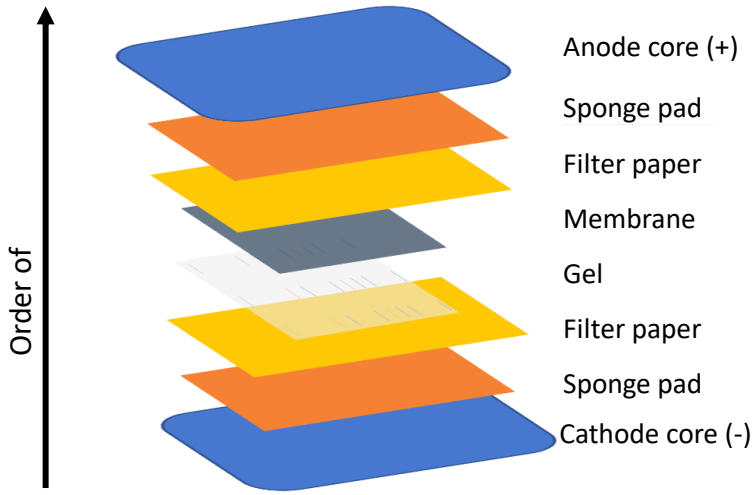


Figure 2.4. Assembling the Mini Blot Module Sandwich. Using the order of assembly as above, set up of the module sandwich was completed ensuring that the membrane was handled with only blotting tweezers. Blotting rollers were used to ensure any air bubbles between the gel, membrane and filter paper were removed (adapted from ThermoFisher manufacturer guidelines).

Once the protein transfer was complete, the membrane was removed from the sandwich and covered in ponceau stain to be left on a rocking shaker at room temperature for 10 minutes.

Following removal of the ponceau stain, the membrane was washed with 1X TBS-T solution. This allowed for the observation of clear, separate lanes of protein bands (confirming that the protein transfer was successful).

2.14.3. Western Blotting

The membrane was blocked by incubating it in milk (10% milk powder dissolved in TBS-T) on a rocking shaker at room temperature for 60 minutes. This was followed by 3 washes in 1X TBS-T, each wash completed for 5 minutes on a rocking shaker, also at room temperature. Using a scalpel, the membrane was then cut along the ladders. Each membrane was added to a 20 mL primary antibody solution (Table 2.2.), made up using TBS-T to a dilution of 1:5,000. Membranes in the antibody solution were then transferred to a cold room (4 – 5 ^{o}C) to incubate overnight.

Following the overnight incubation, membranes were removed from the primary antibody solution and washed again for 3 5-minute in TBS-T. Membranes were then incubated in a 20 - 30 mL secondary antibody solution, also made-up using TBS-T to a dilution of 1:5,000, at room temperature for 60 minutes. Membranes were kept in TBST-T until ready for imaging.

Imaging of the membrane was completed using a ChemiDoc Imaging System (Bio-Rad). Enhanced luminol-based chemiluminescent (ECL) substrate (Clarity ECL, Bio-Rad) was made up to a dilution of 1:1. Each membrane was placed individually on the machine tray using tweezers, ensuring that the tray had been sanitised with 70% ethanol. A white image of the ladder was taken and saved. Exposure time per membrane was adjusted for each antibody accordingly. 200 μL of ECL was then pipetted directly on to the membrane, ensuring that the membrane was fully covered. Imaging was then completed, and the membrane was removed from the tray and placed back into TBS-T, ensuring that the tray was sanitised with 70% ethanol. Imaging was then repeated as above per membrane.

2.14.4. Primary antibodies

When completing the Western blot, the primary antibodies that were used were: anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, a metabolic enzyme that was used as a control), anti-HSPD1 (Hsp60), anti-HSPA1A (Hsp70) and anti-Cytochrome C (Table 2.1.). These antibodies were selected due to their availability within the laboratory and evidence of changes in Hsp60, Hsp70 and Cytochrome C expression following both ethanol and nicotine exposure in other models.

Table 2.2. Primary antibodies.

Antibody	Manufacturer	Ref.	Species	Dilution	Diluted in
GAPDH	Prestige Antibodies	HPA040067	Rabbit	1:5,000	TBS-T
Hsp60	Prestige Antibodies	HPA050025	Rabbit	1:5,000	TBS-T
Hsp70	Prestige Antibodies	HPA052504	Rabbit	1:5,000	TBS-T
Cytochrome C	Cell Signalling Technologies	4272S	Rabbit	1:5,000	TBS-T

ImageJ® was used to analyse the membrane images. Around each protein band, a frame was drawn, ensuring that per membrane, the size of the frame was the same size for each protein band. This generated a curve for each protein band, highlighting the number of active pixels within each frame. The area under the curves was calculated and used to quantify the protein expression within each protein sample. The protein expression for each sample was then made relative to the loading control. Statistical analysis was then completed for each of these values.

2.15. Gas Chromatography – Mass Spectrometry

2.15.1. Fatty acid extraction

Fatty acids were extracted as previously described by Bligh & Dyer (1959).

Ethanol naive worms and ethanol treated worms placed in labelled Eppendorf tubes (WT for wild type for ethanol naive worms and EtOH for ethanol treated worms) with all the pond water removed. Once resuspended in 1 mL of deionised waterm worms were homogenised (Argos Tissue Homogeniser).

To the homogenised samples, 3.75 ml of 1:2 (v/v) chloroform:methanol was added. Once the samples had been vortexed, 1.25 ml of chloroform was added to each sample and the samples were vortexed again. This was repeated once more and the samples were then centrifuged at 1,000 rpm for 5 minutes to separate the chloroform from the water, allowing the observation of an inter-spacial fluff (Figure 2.5.).

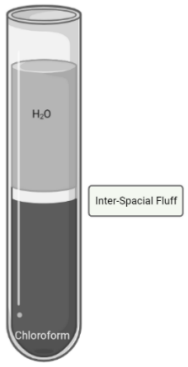


Figure 2.5. Prepared sample, highlighting the inter-spacial fluff. Following centrifugation and the separation of chloroform, methanol and deionised water, the sample should look as above with a layer of chloroform on the bottom. Between the two layers, there should be a layer of inter-spacial fluff which is where the chloroform and water are unable to mix due to being different phases.

Into a fresh GC vial, the chloroform layer was extracted, transferred and samples were dried to complete dryness in a vacuum-centrifuge (SpeedyVac).

2.15.2. Derivatisation

Derivatisation of samples was completed as previously described by Warrilow *et. al* (2016).
In a fume-hood, 200 μL of pyridine was added to each of the samples (to remove pyridine from the bottle, a metal Leur-Lock Syringe with a 115 mm bevel tip SGE Needle was used). Following pyridine, 250 μL of N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA) was pipetted into each of the samples. GC vials were capped, vortexed and heated on a heat block at 100 °C for 30 minutes.
Once samples had been cooled following removal from the heat block, 500 μL of hexane was added to the samples and vortexed.
6 new GC vials were labelled and 500 μL of each sample was transferred to their new GC vials, ready for gas chromatography-mass spectrometry (GC-MS).

2.15.3. Gas Chromatography (GC)

GC analysis was performed with a 6% cyanopropyl/phenyl column (DB 624-UI) (Agilent Technologies). The oven temperature was set follows: initial at 70 °C, with 3 minute hold then ramped to 10 °C /min to 180 °C with 2 minute hold and 10 °C/min to 250 °C with 20 minute hold.
The solvent delay was set 7.8 minutes to avoid the solvent being read at earlier timepoints.

2.15.4. GC-MS analysis

Once the mass spectrum had been generated, data analysis for the GC-MS was carried out using Thermo Xcalibur 2.2 software. By calculating the area of the peaks, the differences in the amount of specific fatty acids between the WT and EtOH samples could be calculated.

2.16. Genetic sequencing and alignments

Protein sequences were acquired using the National Centre for Biotechnology Information (NCBI) protein database. When searching for protein sequences, the receptor name and/or specific subunit and organism name were included, and FASTA sequences were utilised. FASTA amino acid sequences were stored into the CLC Genomics Workbench 22 (Qiagen) and alignments conducted.

Percentage similarities between two or more species protein alignments were completed using the NCBI Basic Local Alignment Tool (BLAST). When the BLAST was run, a percentage of alignment similarity was displayed which was input into a table.

2.17. Acetylcholine quantification

Individual worms were placed in 3 x 24-well plates, with one worm per well and 22 worms per plate, 24 hours prior to extraction. Acetylcholine quantification was completed using manufacturer's guidelines for the MAK056 (SigmaAldrich) kit; this assay uses a colorimetric coupled enzyme reaction to determine the choline concentration. Quantification was done using a spectrophotometer at 570 nm. 10 worms were used to calculate free choline and 10 worms for total choline.

To prepare the *L. variegatus* samples, worms were transferred to centrifuge tubes with all pond water removed. Following the addition of 100 μL of the Choline Assay Buffer, each sample was homogenised and then in a pre-cooled centrifuge at 4^{o}C, samples were spun at 16,100 x g for 10 minutes. 25 μL of the supernatant was used for quantification and any leftover material was discarded.

2.18. Statistical analysis

Statistical analysis for all experiments was completed using GraphPad Prism 9 software.

The sample size for each assay was eight worms unless specified otherwise. Data are displayed as the mean ± standard error of the mean (SEM) for each data set and data are relative to the

untreated control (baseline). When conducting the behavioural assay data analysis, for stereotypical movement assays, paired nonparametric two-tailed *t* tests were used, and for free locomotion assays, paired parametric two-tailed *t* tests were used, with the drug response being compared to the baseline control behaviour for both assays. When analysing both the 10-mins and 24-h rescue time points, two-way ANOVAs with Dunnett's posttest were used with the comparison to baseline controls. Where behavioural response to drug exposure was compared between different drugs, a two-way ANOVA with Dunnett's posttest was used, and statistical differences were again compared to baseline behaviour but also between the two drug exposure responses.

When conducting the *in vitro* experimentation data analysis, a one-way paired student's T-test was used, with the expression levels being compared to the expression of the loading control. The average expression was calculated using three repeats.

For all statistical analysis, $p < 0.05$ was the threshold for significant difference.

2.19. SWIRL collaboration

SWIRL, the laboratory in which this research was conducted, works in a collaborative environment with both postgraduates and undergraduates collecting and analysing data. The author's role has been to plan all the experiments and ensure that where data is collected by another participant, the methodology and data collection has been completed ethically, accurately and in a safe manner. Although data may have been collected collaboratively, data analysis and presentation has been completed by the author. Where data generation has used methods in which there may be inter-individual differences in the scoring, such as for stereotypical movement, it has been the author's responsibility to ensure that when collecting the data, there has been supervision to ensure participants are following the same scoring and during data analysis, to check that there have been enough repeats completed to identify any outliers. Along with the supervisory team, the author's role has also been to manage the laboratory in ensuring that any reagents used are stored safely and where needed, are handled with the correct procedure.

3. Results Chapter 1: Ethanol

3.1. Investigating ethanol and *L. variegatus* behaviour

The mechanism of action of ethanol is contended but administration of ethanol to invertebrate models, such as *C. elegans*, has resulted in a dose-dependent decrease in locomotory behaviour (Mitchell et al., 2007).

Knowing that *L. variegatus* previously exposed to increasing concentrations of 0 – 500 mM ethanol demonstrated a dose-dependent decrease in both stereotypical and free locomotory behaviour (Bellamy, 2023) (Appendix Figure 1), we wanted to determine the onset of action at which ethanol began to inhibit free locomotory motion in *L. variegatus*.

Figure 3.1. shows that during 10-mins exposure to 250 mM and 500 mM ethanol, a significant decrease in free locomotory movement begins at 2-mins, where the area covered by *L. variegatus* decreased by 35.59%±6.45% ($p=0.0194$) during 250 mM ethanol exposure and decreased by 33.10%±8.70% ($p=0.0330$) during 500 mM ethanol exposure. This significant decrease in movement was observed at 2 minutes and all timepoints throughout the 10-mins exposure at 250 mM and 500 mM ethanol ($p<0.05$).

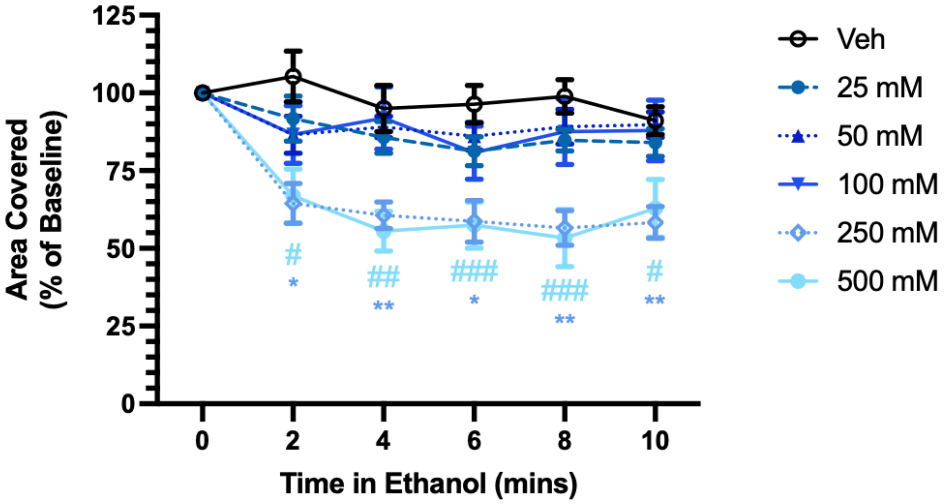


Figure 3.1. Onset of action of ethanol in *Lumbriculus variegatus* between 0 and 10 minutes of exposure. *L. variegatus* were exposed to increasing concentrations of ethanol (0 – 500 mM) for 10 minutes. Rapid images were collected at 2-minutes time points. Each time point was compared to the pre-exposure (0 minutes). n=11 technical replicates over two experimental replicates. *shows statistical significance for 250mM ethanol, # shows the statistical significance for 500mM ethanol. */# p<0.05, **/## p<0.01, ### p<0.001. (Data was generated by Elis Roome and Kwang Lee and analysed by Romessa Mahmood).

Observing that the inhibitory effect on *L. variegatus* behaviour following 10-mins exposure to ≥250 mM ethanol is not observed at ≤50 mM ethanol concentrations, (Bellamy, 2023) (Appendix Figure 1), we sought to examine the effects of lower concentrations of ethanol when administered *to L. variegatus*. Concentrations as low as 1-10 mM ethanol have been shown to produce an excitatory effect within synaptic currents (Harrison et al. 2017).

No excitatory effect following administration of 0 - 50 mM ethanol was observed as there was no significant change in both *L. variegatus* stereotypical (Figure 3.2. A & B) and free locomotory movement (Figure 3.2. F). Recovery of *L. variegatus* following the removal of ethanol also showed no significant difference when compared to baseline for both stereotypical (Figure 3.2. C & D) and free locomotory movement (Figure 3.2. G).

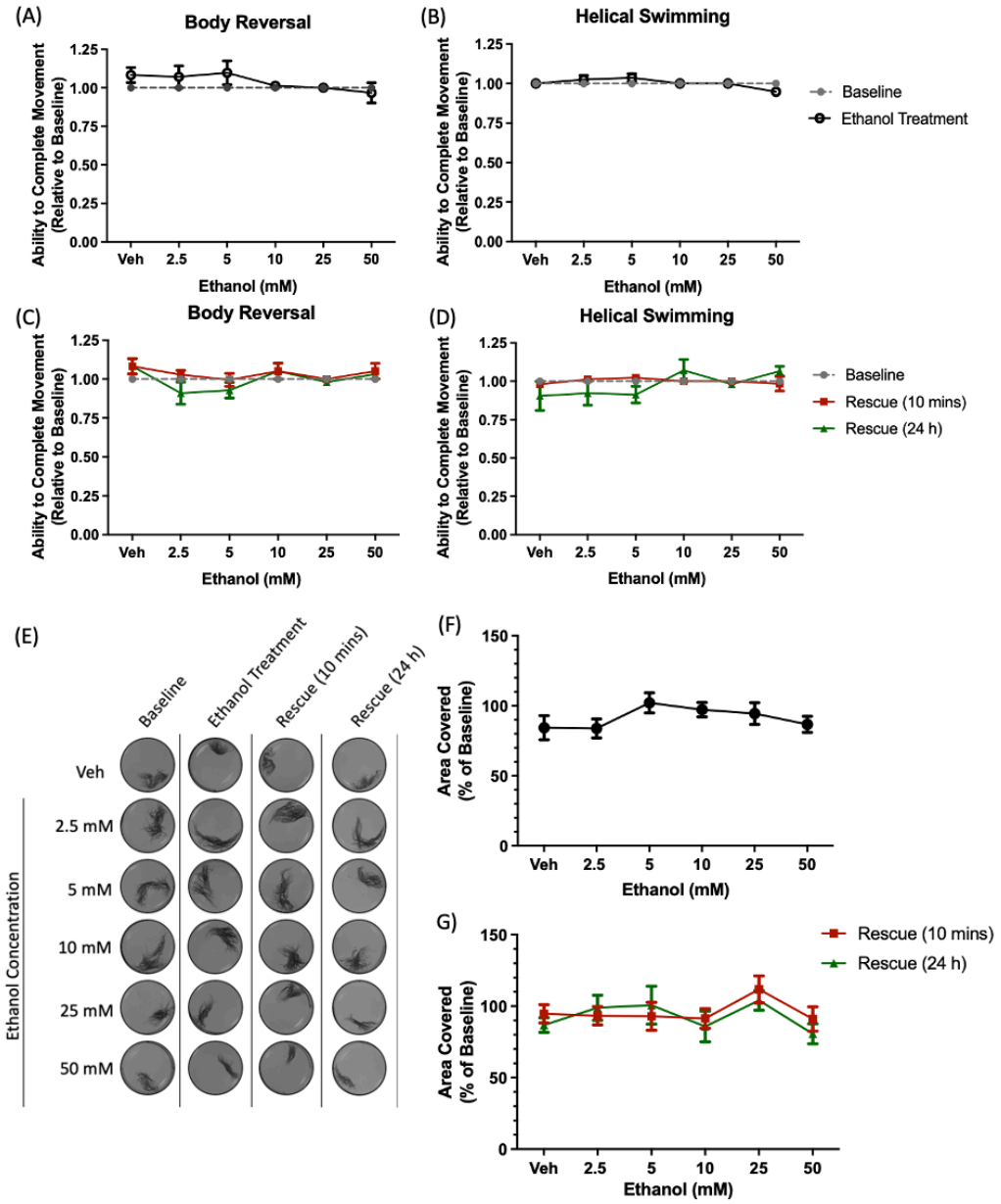


Figure 3.2. The effect of ethanol on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of ethanol (0 – 50 mM) and tested for the ability of tactile stimulation to elicit (A) body reversal or (B) helical swimming. Ethanol was then removed and the ability of *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 0 – 50 mM ethanol, 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 expressed as a percentage of the area covered at baseline. Data is presented as a percentage of the area of baseline movement. Error bars represent the standard error of the mean, n=8 experimental replicates for each concentration. Veh: artificial pondwater (data was generated in collaboration with Elis Roome and Kwang Lee and data analysis was completed by Romessa Mahmood).

As short-term effects of ethanol exposure had been observed *in vivo*, Western blotting was completed to observe the short-term effects of ethanol on *L. variegatus in vitro* on the expression of Hsps and cytochrome C (Figure 3.3.). Heat shock proteins 70 (Hsp70) and 60 (Hsp60) and cytochrome C can all be used as markers for toxicity.

We observed that *L. variegatus* exposed to 500 mM ethanol expressed significantly increased Hsp70 levels by 1.89±0.25 ($p=0.0169$) (Figure 3.3. B), when compared to *L. variegatus* exposed to artificial pondwater. Ethanol exposure did not, however, alter the expression of Hsp60 and cytochrome C ($p>0.05$) when compared to the expression of both in *L. variegatus* exposed to artificial pondwater.

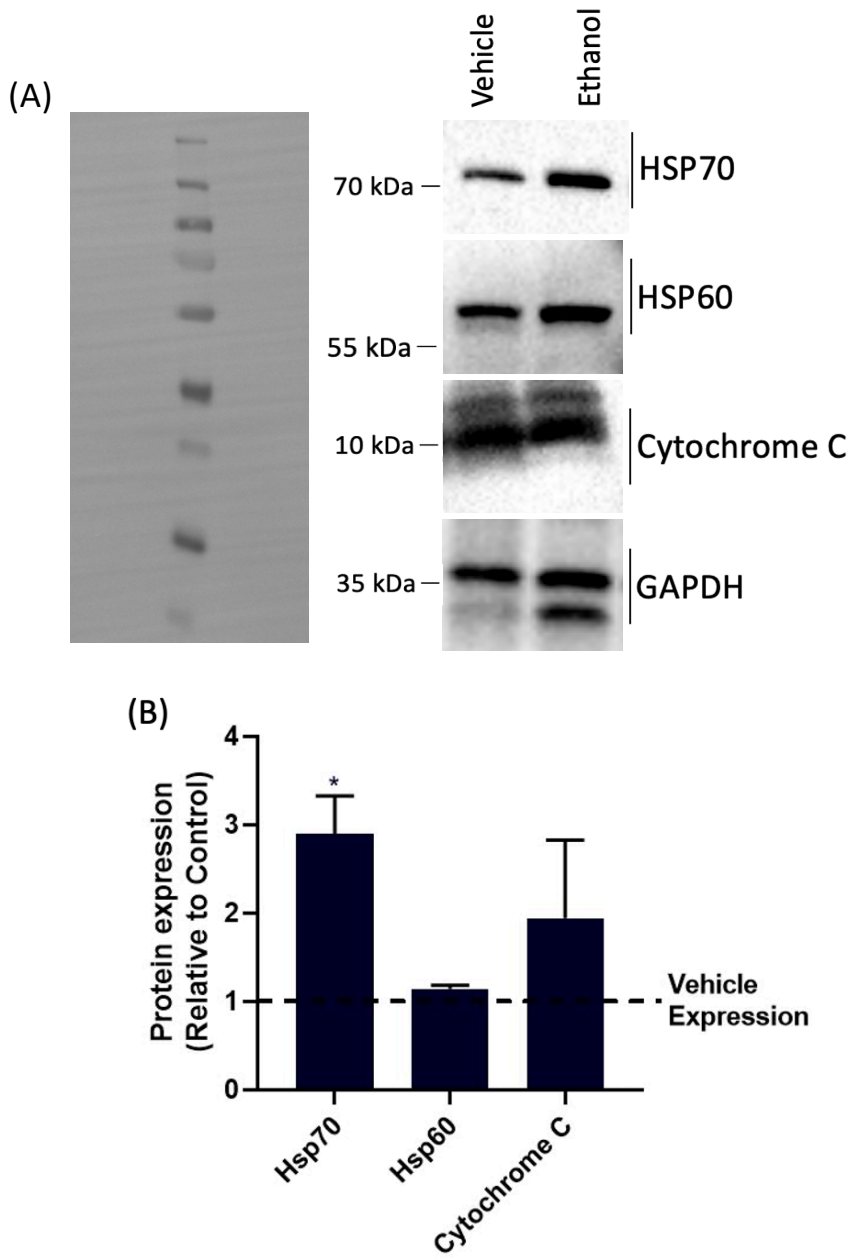


Figure 3.3. Western Blotting of Hsp60, Hsp70 and Cytochrome C expression in vehicle-treated and ethanol-treated *Lumbriculus variegatus*. Prior to protein extraction, *L. variegatus* were treated in either artificial pondwater or 500 mM ethanol for 10 minutes. *L. variegatus* protein samples were prepared and run through 8 – 12% SDS-PAGE gel. **(A)** Western blotting was completed using anti-HSPA1A, anti-HSPD1 and anti-Cytochrome C antibodies. GAPDH was used as a loading control. **(B)** Densitometry analysis quantified the Western blot of protein expression in *L. variegatus* exposed to 500 mM ethanol relative to protein expression in control *L. variegatus*, "Vehicle", exposed to artificial pondwater. Protein expression was normalised to GAPDH loading control. Error bars represent the standard error of the mean from three experimental replicates. Statistical analysis was carried out using a one-way paired Student's t-test, *$p<0.05$ (data was generated in collaboration with Julanta Carriere and analysed by Romessa Mahmood).

Having observed the short-term response to ethanol both *in vitro* and *in vivo*, we optimised an assay to observe whether *L. variegatus* developed an acute tolerance to ethanol.

Figure 3.4. A demonstrates the effects of continuous exposure to 500 mM ethanol at 20 minutes timepoints over the 210-mins. Figure 3.4. B shows that following 10-mins exposure to 500 mM ethanol, the area covered by *L. variegatus* decreased to 41.99%±3.85% ($p<0.0001$). Movement then significantly decreased further at 30 minutes to 25.62%±3.35% ($p=0.0026$) and at 50 minutes to 24.85%±4.49% ($p=0.0049$). At 70 minutes, movement returned to 10-mins exposure levels, increasing to 45.87%±6.10%, with no significant difference compared to the 10-minute timepoint ($p>0.05$). Movement then significantly increased to 57.63%±4.42% ($p=0.0428$) at 150 minutes and to 60.58%±4.23% ($p=0.0390$) at 210 minutes.

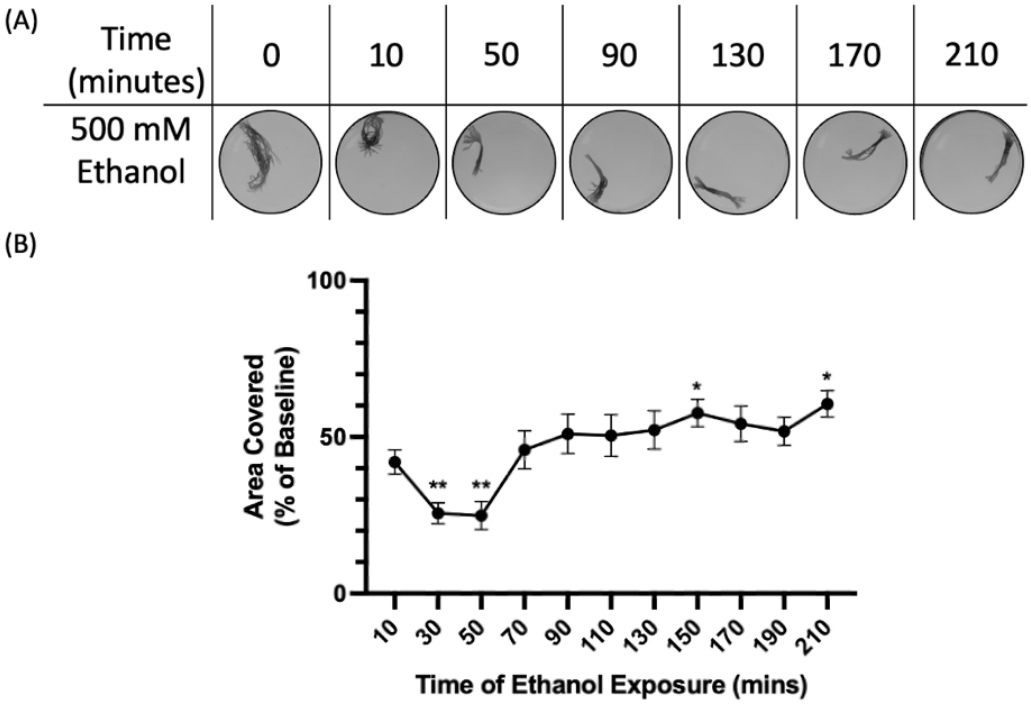


Figure 3.4. The demonstration of acute functional tolerance in *Lumbriculus variegatus* during 500mM ethanol exposure. (A) *L. variegatus* free locomotion was measured after 10 minutes of exposure to 500mM of ethanol and then at 20-mins intervals for 210 minutes. **(B)** Quantification of *L. variegatus* free locomotion expressed as a percentage of free locomotion before ethanol exposure. Statistical differences were measured by one-way ANOVA with Dunnett's post-hoc test compared to the 10 minutes ethanol exposure, *$p<0.05$, **$p<0.01$. Error bars represent the standard error of the mean. n=12 technical replicates over two experimental replicates.

As we had observed that *L. variegatus* developed acute tolerance at 500 mM over a timepoint of 210-mins, we sought to investigate the effects of ethanol following chronic exposure to ethanol by determining the lethal dose for 50% of the *L. variegatus* population of ethanol (LD_{50}) over a timepoint of 72 hours.

We observed that over 72 hours, exposure to increasing concentrations of ethanol caused no lethality of *L. variegatus* (Figure 3.5.). Due to a 100% survival rate, the LD_{50} for ethanol in *L. variegatus* could not be established.

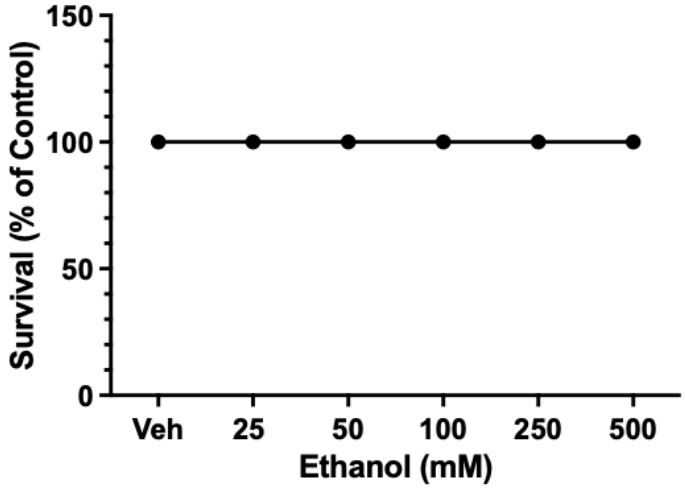


Figure 3.5. Determining the LD50 of ethanol in *Lumbriculus variegatus*. *L. variegatus* were exposed to increasing concentrations of ethanol (0 – 500 mM) for 72 hours. Every 24 hours, the number of *L. variegatus* alive was counted and recorded. Data was generated in collaboration with Elis Roome and Kwang Lee and data analysis was completed by Romessa Mahmood. n = 6 experimental replicates. No statistical significance was found.

As no toxicity for ethanol exposure was observed in Figure 3.5., we cultured *L. variegatus* in 100 mM ethanol for ≥ 21 days as we had observed this concentration has no significant effects on *L. variegatus* behaviour (Appendix Figure 1). *L. variegatus* from this culture were exposed to increasing concentrations of ethanol to determine whether they developed chronic tolerance to ethanol.

Figure 3.6. A & B highlights that whilst significant decreases in locomotory movement were observed at ≥100 mM ethanol for both *L. variegatus* that were cultured under normal conditions (ethanol-naïve) and 100 mM ethanol-cultured *L. variegatus*, there was no statistical differences between the two *L. variegatus* groups in their decreased response. At 500 mM ethanol, the area covered by ethanol naïve *L. variegatus* decreased by 44.43%±3.97% ($p<0.0001$) from baseline and the area covered by ethanol cultured *L. variegatus* decreased by 48.35%±6.42% ($p=0.0001$) from baseline. Both groups demonstrated the previously observed recovery in movement back to baseline 10-mins and 24 hours post ethanol removal (Figure 3.6. C and D respectively).

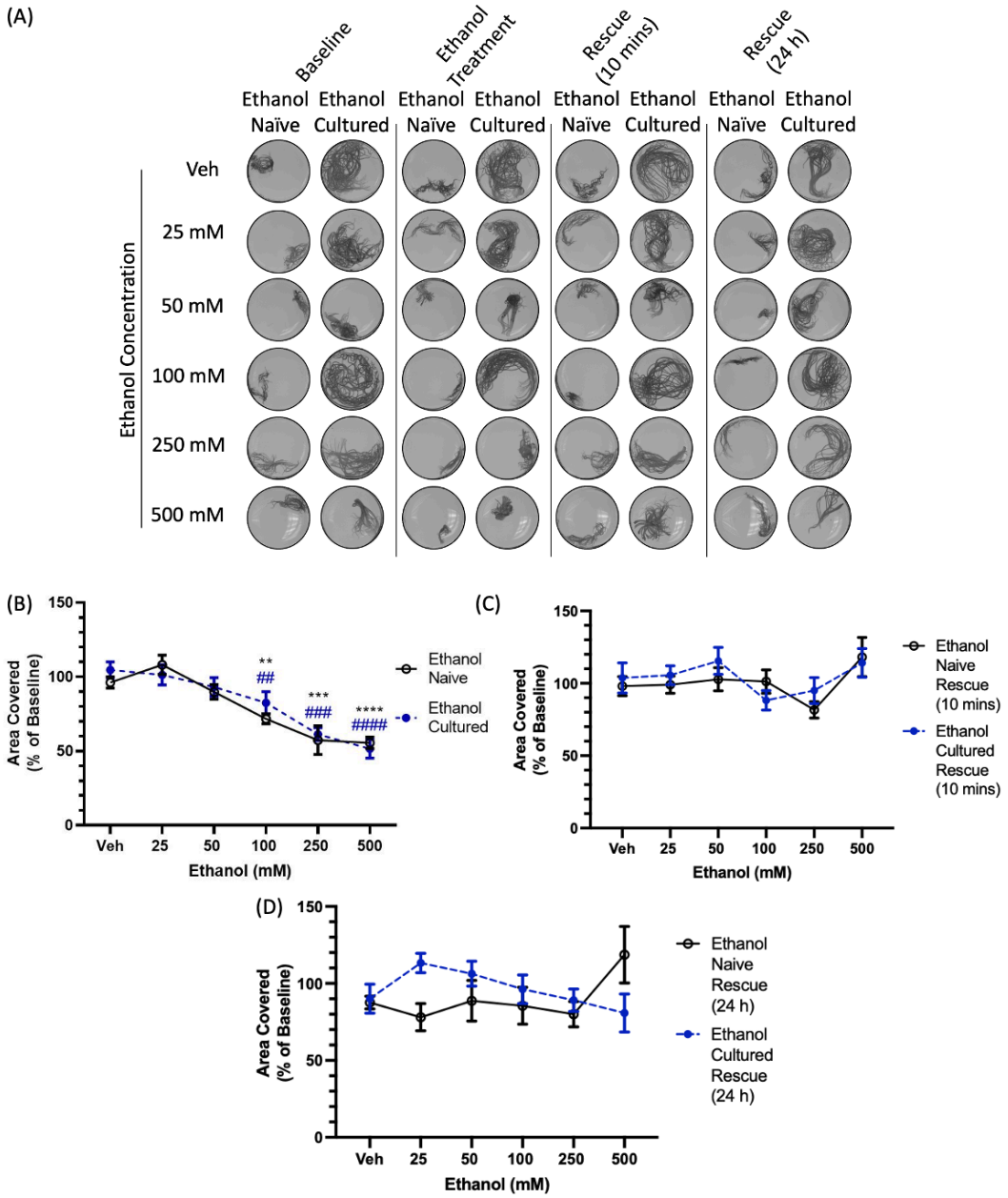


Figure 3.6. Comparison of the effect of ethanol on ethanol-naïve to ethanol-treated *Lumbriculus variegatus* free locomotory behaviour. *L. variegatus* were exposed to increasing concentrations of ethanol (0 – 500 mM). **(A)** The effect of ethanol on free locomotion was measured before ethanol exposure (Baseline), after 10 minutes of exposure to 0 – 500 mM ethanol, 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 **(B)** ethanol treatment and **(C)** removal of ethanol for 10 minutes and **(D)** 24 hours are expressed as a percentage of the area covered at baseline. Statistical differences were measured by parametric Student's t-tests were used to compare ethanol naïve to ethanol cultured *L. variegatus* at each concentration. Error bars represent the standard error of the mean, n=8 experimental replicates for each concentration. *Shows statistical significance for ethanol naive, # shows the statistical significance for ethanol cultured. **/## p<0.01, ### p<0.001. Veh: artificial pondwater, (data was generated in collaboration with Elis Roome and Kwang Lee and data analysis was completed by Romessa Mahmood).

It has not been demonstrated in previous studies that chronic ethanol consumption can result in changing the physical size of the model and therefore we sought to determine whether there was a significant difference in the body sizes of ethanol naïve and ethanol cultured *L. variegatus*. We also wanted to observe further as to whether the difference in physical sizes affected the movement of *L. variegatus*.

Culturing *L. variegatus* in 100 mM ethanol resulted in a significant increase in body size in comparison to culturing *L. variegatus* in artificial pond water alone ($p=0.0002$) (Figure 3.7.A). Where ethanol naïve *L. variegatus* had an average body size of 0.38 $cm^2 \pm 0.03$ cm^2, ethanol cultured *L. variegatus* had a significantly increased average body size of 1.09 $cm^2 \pm 0.12$ cm^2 ($p=0.0002$).

Figure 3.7. B demonstrates, however, that this significant increase in body size of ethanol cultured *L. variegatus* does not result in a significant difference in movement between ethanol naïve and ethanol cultured *L. variegatus* relative to body size ($p>0.05$).

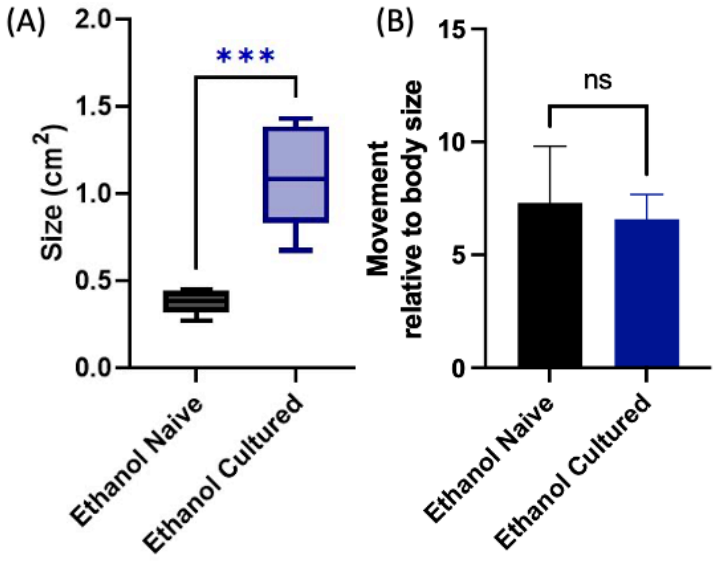


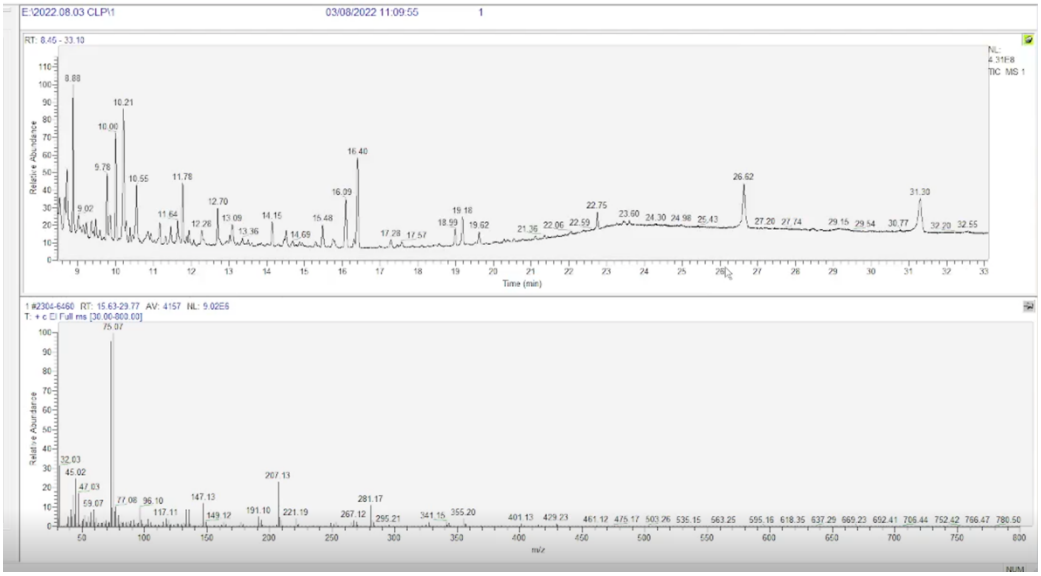
Figure 3.7. Comparison of ethanol naïve vs ethanol cultured *Lumbriculus variegatus* (A) body size and (B) movement relative to body size. *L. variegatus* cultured in artificial pond water had their body sizes compared to *L. variegatus* cultured in 100 mM ethanol. Data was collected by using an adapted free locomotion assay method, where an individual image was used to calculate the size. Using the size, the equation from 2.10. was used to calculate movement relative to body size. Statistical analysis was completed using a paired t-test. n=6 technical replicates, ***p<0.001 (data was generated in collaboration with Elis Roome data analysis was completed by Romessa Mahmood).

As we observed that whilst chronic ethanol administration did not alter *L. variegatus*' behavioural response to acute ethanol administration, it did cause a change in body size and therefore we aimed to explore whether there were any physiological changes that could be observed *in vitro*.

After fatty acid samples, isolated from both ethanol-naïve and ethanol cultured *L. variegatus*, were run through the GC-MS, we were able to observe the presence of stearic acid and palmitic acid in both samples. Oleic acid was also present in the ethanol cultured samples but not in the ethanol-naïve samples. Between the ethanol-naïve and ethanol cultured samples, there was no significant increase observed for both stearic acid and palmitic acid ($p>0.05$).

Table 3.1. Area of peaks for fatty acids observed in ethanol naïve and ethanol cultured *Lumbriculus variegatus.* (A) Fatty acid samples isolated from both ethanol naïve and ethanol cultured *L. variegatus* were run through the GC-MS. **(B)** The average areas of the peaks were calculated using Thermo Xcalibur, n=3 for each sample.

(A)



(B)

Fatty Acid	Area of Peak (Abundance-minutes)	
	Ethanol Naïve *L. variegatus*	Ethanol Cultured *L. variegatus*
Stearic Acid	2006392	3231089
Palmitic Acid	2000611	2681473
Oleic Acid	-	1358197

3.2. Investigating GABAergic pathways in *L. variegatus*

As the $GABA_A$ and $GABA_B$ pathways have been suggested as potential target receptors for the mechanism of action of ethanol, we sought to investigate the were conservation of these receptors across species.

$GABA_A$ receptors are most commonly composed of three subunits: α1, β2 and γ2 (Schwartz, 1988). $GABA_B$ receptors have two subunits: subunit 1 and subunit 2. Using each subunit alignment (Appendix Figure 2-4), the *Homo sapiens* alignment for each subunit was compared to the alignments for each of the other species. Percentage similarities were recorded in Table 3.1., highlighting the conservation of each subunit from human to *C. elegans*. *L. variegatus* is not yet sequenced and not included in this analysis.

Table 3.1. shows that $GABA_A$ receptor subunit homologs are observed in *C. elegans*. *C. elegans* have not been shown to express a homologous $GABA_A$ receptor pathway. Whilst *Xenopus laevis* tadpoles have been shown to exhibit a $GABA_A$ receptor pathway (Reith & Sillar, 1999), the $GABA_A$ receptor β2 subunit was not shown to be conserved down to *Xenopus* when running the alignments.

Table 3.2. Conservation of $GABA_A$ and $GABA_B$ receptor subunits from human to genus. Alignments of $GABA_A$ receptor subunits α1, β2 and γ2 and $GABA_B$ receptor subunits 1 and 2 were run for *Homo sapiens*, *Rattus*, *Mus*, *Xenopus* and *Caenorhabditis*. Using BLAST, the percentage similarities between human alignments and other species were compared to calculate the conservation of the subunits across species.

Human Protein	Conservation of Human to Genus			
	Rattus	*Mus*	*Xenopus*	*Caenorhabditis*
GABAA Receptor α1	98.25%	93.86%	88.11%	-
GABAA Receptor β2	99.79%	99.79%	-	-
GABAA Receptor γ2	98.72%	97.05%	90.16%	-
GABAB Receptor subunit 1	98.75%	98.02%	-	43.28%
GABAB Receptor subunit 1	98.30%	98.41%	87.54%	37.61%

As we demonstrated that $GABA_A$ receptor pathways were not conserved down to *C. elegans,* but the $GABA_B$ receptor pathways were, the effects of GABA and its receptor antagonists and agonists, that are currently used in alcohol dependent treatments, were explored regarding *L. variegatus* behaviour.

Previous experiments showing the behavioural impact of GABA (Appendix Figure 5) and bicuculline (Appendix Figure 6) alone were conducted. These demonstrated that the NOAEL of GABA was 1mM and the NOAEL of bicuculline was 2.5 mM. We therefore pre-treated *L. variegatus* with 2.5 mM bicuculline and then administered increasing concentrations of GABA.

Figure 3.8. A and B respectively show that pre-treatment of bicuculline did not significantly impact *L. variegatus* body reversal or helical swimming during 10-mins exposure to GABA compared to the administration of GABA alone ($p>0.05$). Figure 3.8. C-F demonstrates that this is also seen 10 minutes and 24 hours following GABA removal ($p>0.05$).

Figure 3.9. B shows that pre-treatment of bicuculline also did not significantly impact *L. variegatus* free locomotory behaviour during 10-mins exposure to GABA compared to the administration of GABA alone ($p>0.05$). At 100 mM GABA, the average area covered by *L. variegatus* when exposed to GABA alone decreased by 42.27%±10.95% whereas when pre-treated with bicuculline, the average area covered decreased by 36.02%±13.68%. Figure 3.9. C and D demonstrates that this is also seen 10 minutes and 24 hours following GABA removal.

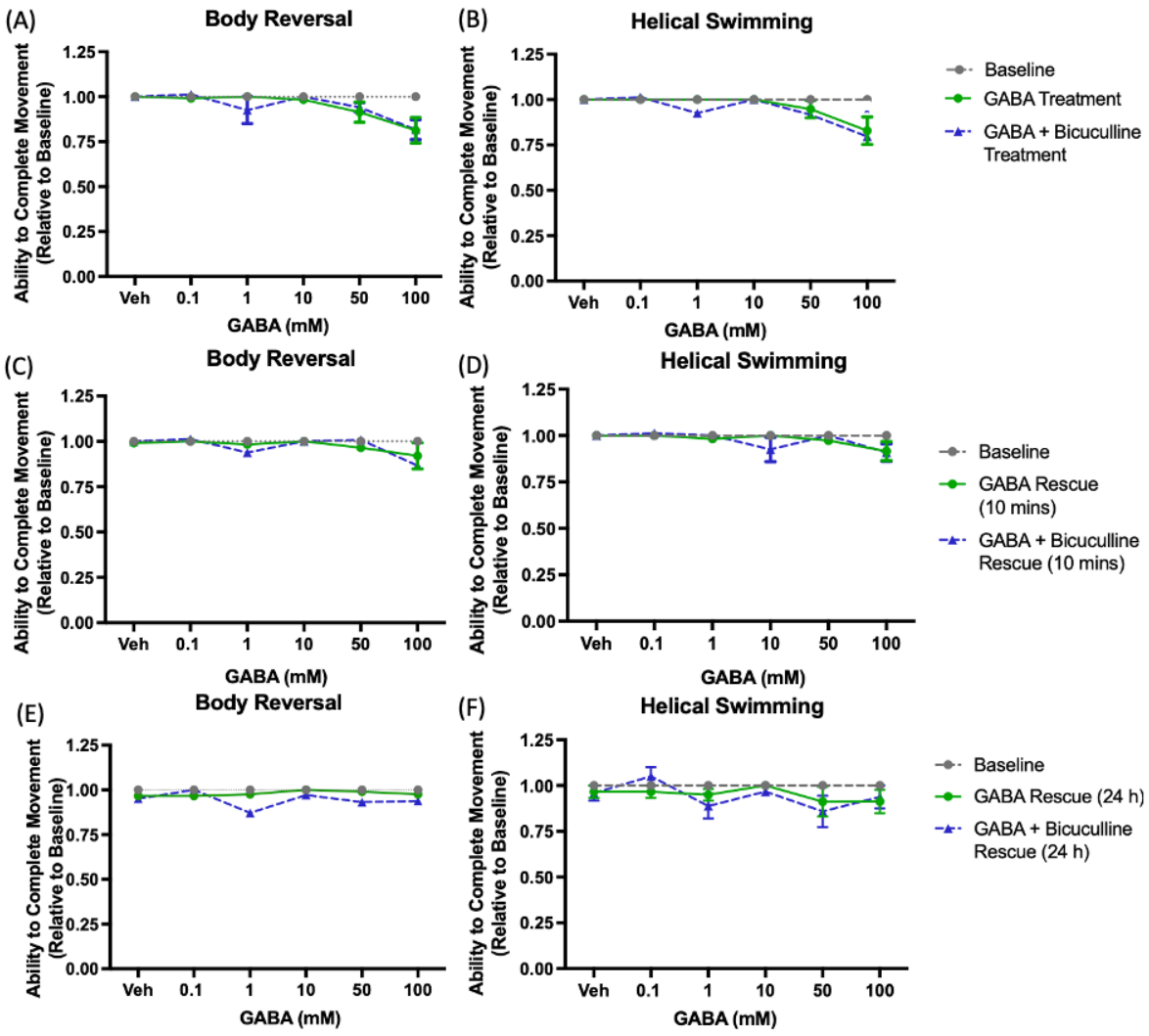


Figure 3.8. Comparison of the effects of GABA alone and GABA + bicuculline on *Lumbriculus variegatus* behaviour. *L. variegatus* were pre-treated with either artificial pondwater or 2.5 mM bicuculline for 10 minutes. Following removal of pre-treatment, *L. variegatus* were exposed to increasing concentrations of GABA (0 – 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 **(E, F)** 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline, n=8 experimental replicates for each concentration. Veh: artificial pondwater.

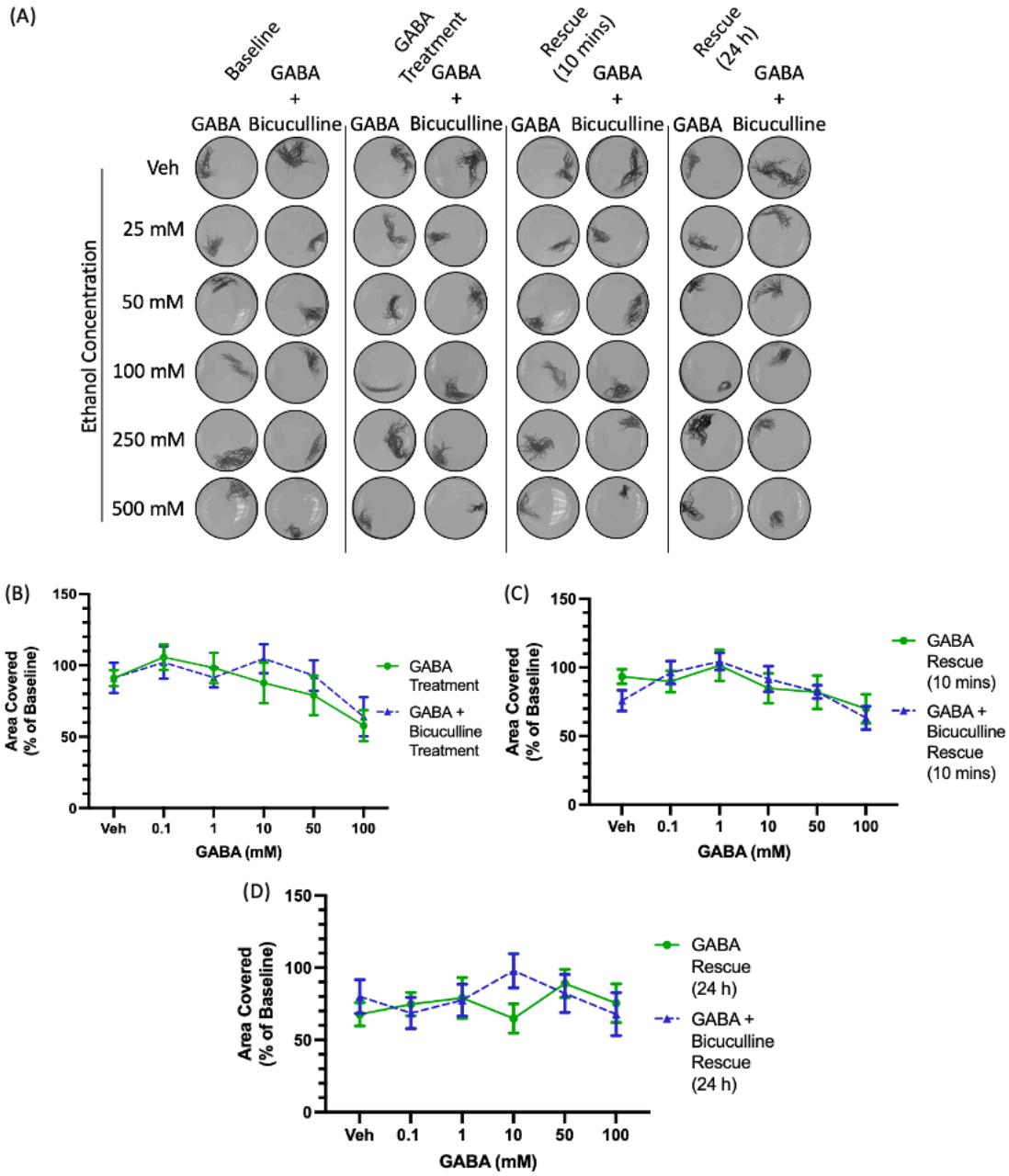


Figure 3.9. Comparison of the effects of GABA alone and GABA + bicuculline on *Lumbriculus variegatus* behaviour. (A) The effect of GABA on free locomotion was measured before GABA exposure (Baseline), after 10 minutes of exposure to 0 – 100 mM GABA, 10 minutes after GABA removal (Rescue (10 mins)) and 24 hours after GABA removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(B)** GABA treatment and removal of GABA for **(C)** 10 minutes and **(D)** 24 hours are expressed as a percentage of the area covered at baseline. n=8 for each concentration. Statistical differences were measured by two-way ANOVA. Error bars represent the standard error of the mean, n=8 experimental replicates for each concentration. Veh: artificial pondwater.

To further investigate whether GABA alters the effects of ethanol in *L. variegatus*, *L. variegatus* were pre-treated with either 1 mM GABA or 2.5 mM bicuculline and then treated with increasing concentrations of ethanol.

Figure 3.10. B highlights that the pre-treatment of GABA or bicuculline with ethanol does not significantly impact *L. variegatus* free locomotory behaviour in comparison to ethanol alone, apart from at 100 mM ethanol + bicuculline. At 100 mM ethanol, the average area covered by *L. variegatus* when exposed to ethanol alone decreased by 43.97%±6.04% whereas when pre-treated with bicuculline, the average area covered decreased by 5.86%±5.63% ($p=0.0027$).

Figures 3.10. C-D show that movement of *L. variegatus* returned to baseline levels for all concentrations of ethanol 10 minutes and 24 hours post ethanol removal.

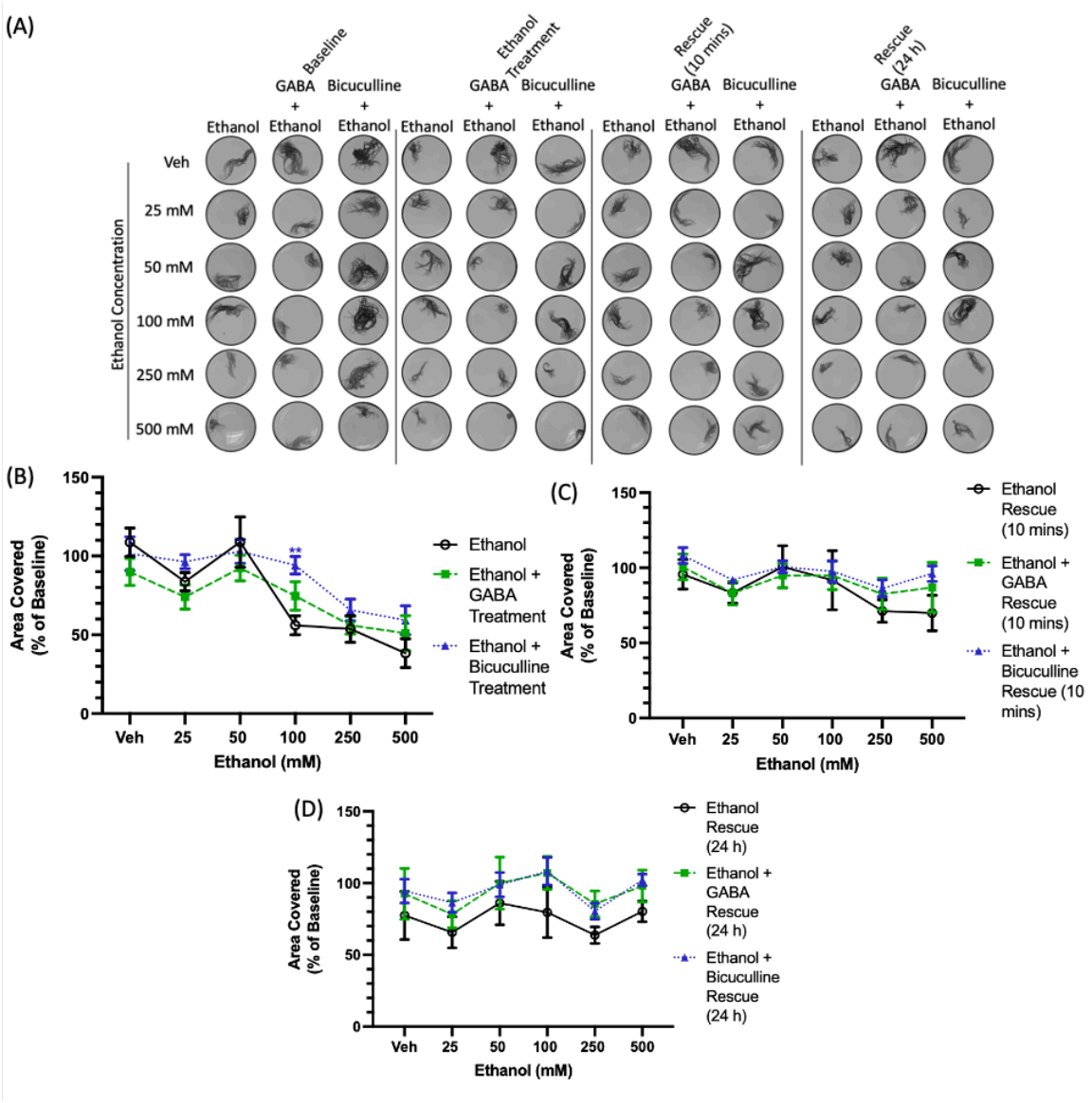


Figure 3.10. Comparison of the effects of ethanol alone, ethanol + GABA and ethanol + bicuculline on *Lumbriculus variegatus* free locomotory behaviour. *L. variegatus* were pre-treated with either artificial pondwater, 1 mM GABA or 2.5 mM bicuculline for 10 minutes. Following removal of pre-treatment, *L. variegatus* were exposed to increasing concentrations of ethanol (0 – 500 mM). **(A)** The effect of ethanol on free locomotion was measured before ethanol exposure (Baseline), after 10 minutes of exposure to 0 – 500 mM ethanol, 10 minutes after removal of ethanol and 24 hours after removal of ethanol. Quantification of the area covered by *L. variegatus* following **(B)** ethanol treatment and removal of ethanol for **(C)** 10 minutes and **(D)** 24 hours are expressed as a percentage of the area covered at baseline. Statistical differences were measured by two-way ANOVA. Error bars represent the standard error of the mean, n=8 experimental replicates for each concentration. **p<0.01. Veh: artificial pondwater.

As we were unable to establish the presence of a $GABA_A$ receptor pathway in *L. variegatus*, we sought to explore the possibility of the expression of a $GABA_B$ receptor pathway in *L. variegatus*. *L. variegatus* were exposed to increasing concentrations of baclofen, a $GABA_B$ receptor agonist.

Figure 3.11. A-B show that 10-mins exposure to increasing concentrations of baclofen resulted in no significant change in both body reversal and helical swimming ($p>0.05$) compared to baseline movements ($p>0.05$), with no significant effects observed 10 minutes and 24 hours following baclofen removal (Figure 3.11. C-D, $p>0.05$).

Figure 3.11. F shows that 10-mins exposure to increasing concentrations of baclofen also resulted in no significant change in *L. variegatus* free locomotory movement compared to baseline ($p>0.05$) and, as with stereotypical movements, there were no effects observed 10 minutes and 24 hours following baclofen removal (Figure 3.11. G, $p>0.05$).

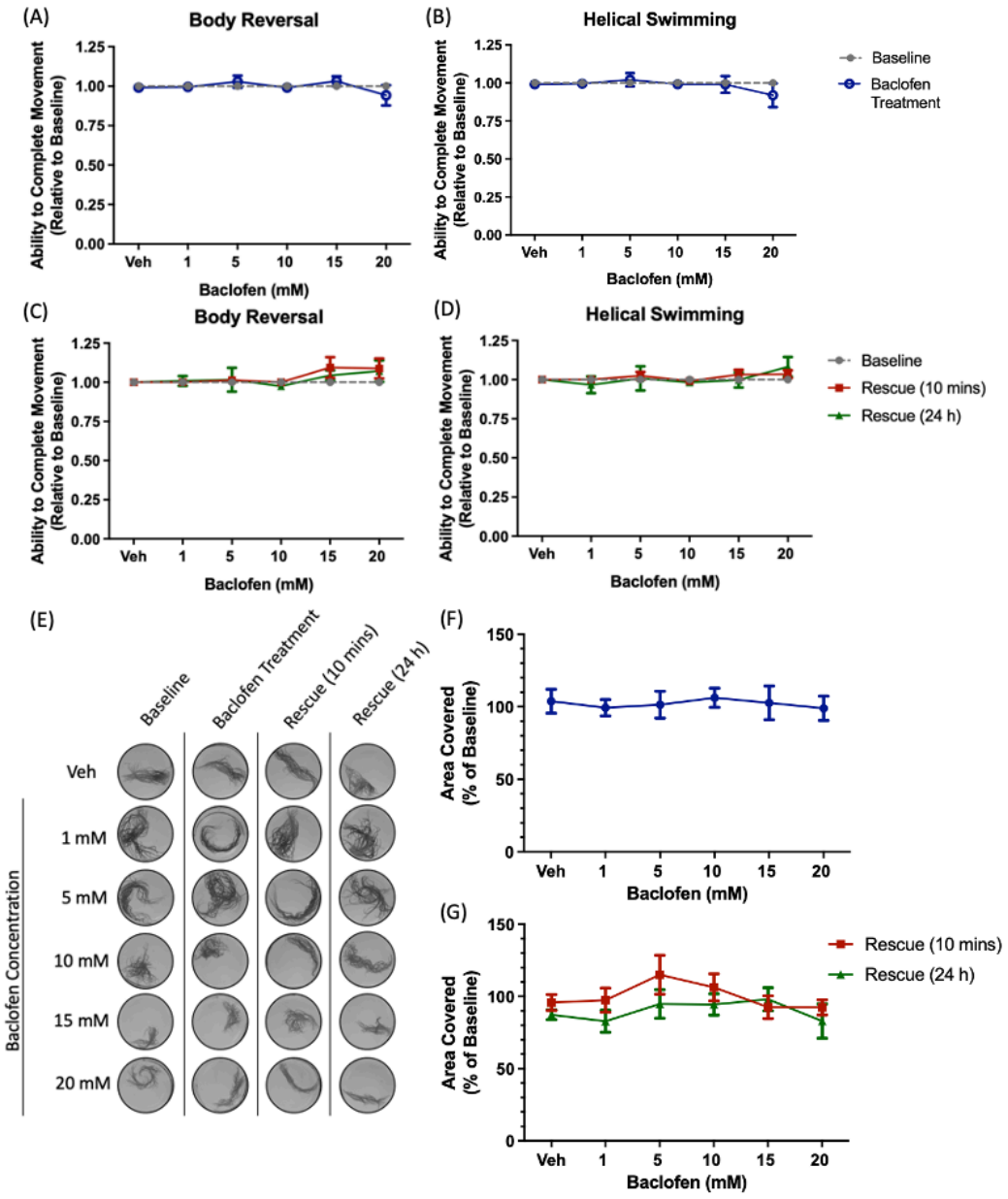


Figure 3.11. The effect of baclofen on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of baclofen (0 – 20 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Baclofen was then removed and the 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 baclofen on free locomotion was measured before baclofen exposure (Baseline), after 10 minutes of exposure to 0 – 20 mM baclofen, 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)** baclofen treatment and **(G)** removal of baclofen for 10 minutes and 24 hours are expressed as a percentage of the area covered at baseline, n=8 experimental replicates for each concentration. Veh: artificial pondwater.

To further investigate the presence of a $GABA_B$ receptor in *L. variegatus* and its role in the mechanism of action of ethanol, *L. variegatus* were pre-treated with 20 mM baclofen, which was shown to have no effect on stereotypical movement or free locomotion (Figure 3.12) and then administered increasing concentrations of ethanol (0-500 mM).

Figure 3.12. A-B shows that at 100 mM ethanol, the pre-treatment of 20 mM baclofen prevents a significant reduction in body reversal ($p=0.0227$) and helical swimming ($p=0.0223$). At 250 mM and 500 mM ethanol, however, pre-treatment of 20 mM baclofen appears to result in no change in the significant decrease in movement caused by ethanol exposure ($p>0.05$). Figure 3.12. C-F shows that body reversal and helical swimming return to baseline levels 10-mins and 24 hours following ethanol removal.

Figure 3.13. B, however, does not show that the pre-treatment of 20 mM baclofen prevents a significant reduction in free locomotory movement at any concentration of ethanol. At 500 mM ethanol, the average area covered by *L. variegatus* when exposed to ethanol alone was reduced by 62.12%±8.37% and when pre-treated with baclofen, the average area covered was reduced by 69.99%±6.81%. Figure 3.13. I-J shows that body reversal and helical swimming return to baseline levels 10-mins and 24 hours following ethanol removal.

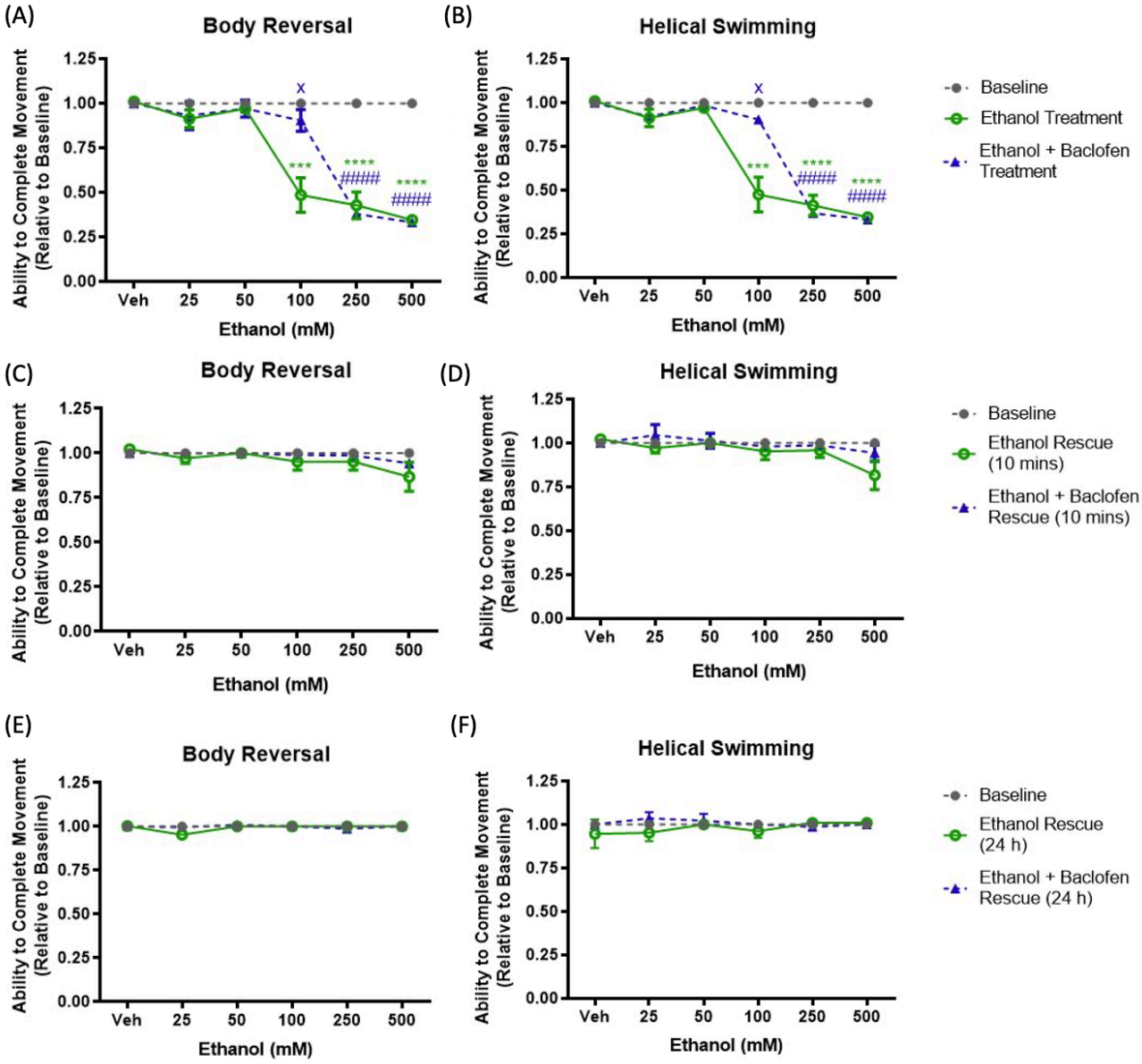


Figure 3.12. Comparison of the effects of ethanol alone and ethanol + baclofen on *Lumbriculus variegatus* behaviour. *L. variegatus* were pre-treated with either artificial pondwater or 20 mM baclofen for 10 minutes. Following removal of pre-treatment, *L. variegatus* were exposed to increasing concentrations of ethanol (0 – 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 **(E, F)** 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline, n=8 experimental replicates for each concentration. Veh: artificial pondwater. *Shows statistical significance for ethanol relative to baseline, # shows the statistical significance for ethanol + baclofen relative to baseline, x shows statistical significance for ethanol + baclofen relative to ethanol. xp<0.05, ***/###p<0.001, ****/####p<0.0001 (data was generated in collaboration with Gemma Rees, data analysis was completed by Romessa Mahmood).

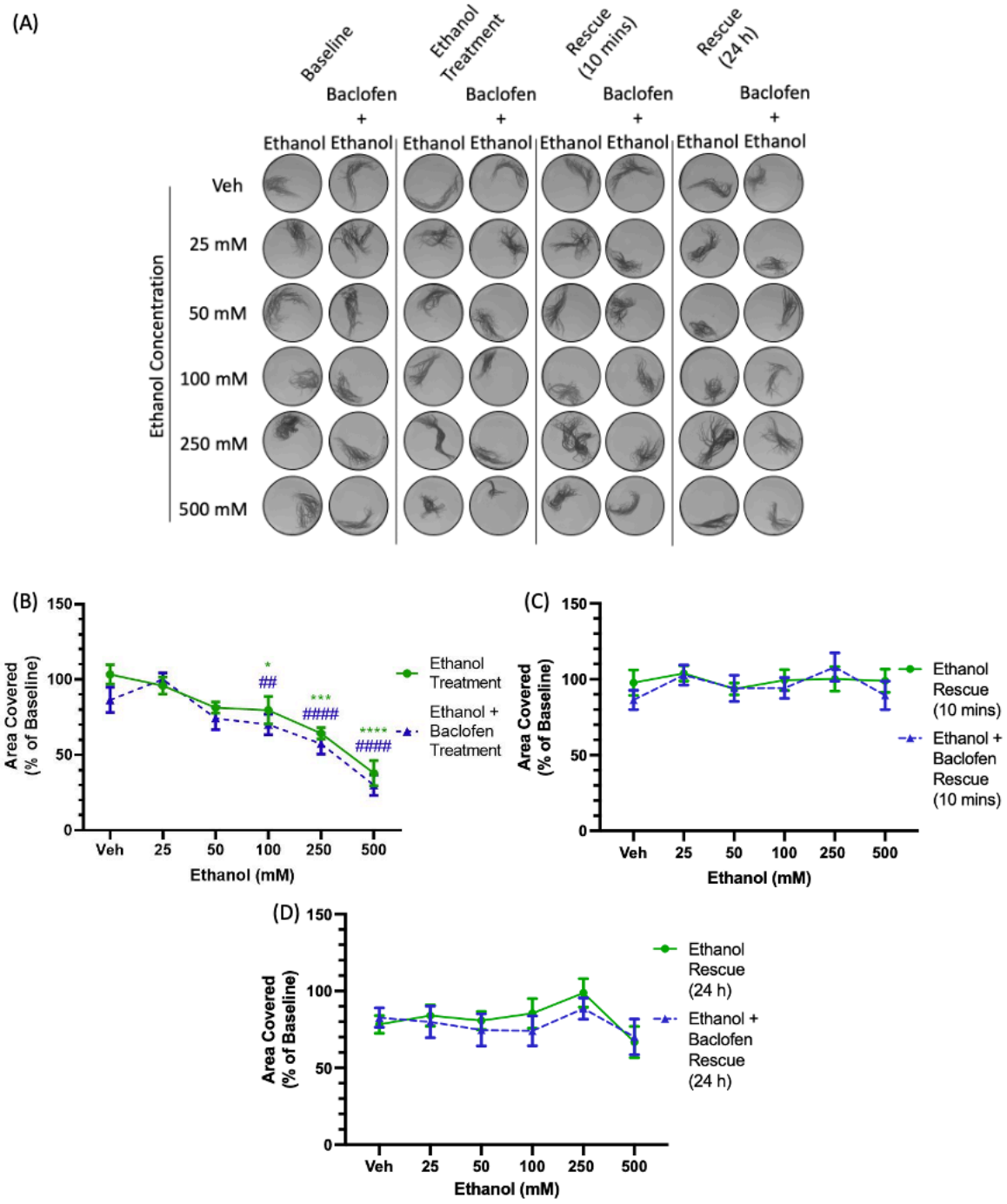


Figure 3.13. Comparison of the effects of ethanol alone and ethanol + baclofen on *Lumbriculus variegatus* behaviour. (A) The effect of ethanol on free locomotion was measured before ethanol exposure (Baseline), after 10 minutes of exposure to 0 – 500 mM ethanol, 10 minutes after ethanol removal (Rescue (10 mins)) and 24 hours after ethanol removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(B)** ethanol treatment and removal of ethanol for **(C)** 10 minutes and **(D)** 24 hours are expressed as a percentage of the area covered at baseline, n=8 experimental replicates for each concentration. Veh: artificial pondwater, *shows statistical significance for ethanol, # shows the statistical significance for ethanol + baclofen, *p<0.05, ***/###p<0.001, ****/####p<0.0001 (data was generated in collaboration with Gemma Rees, data analysis was completed by Romessa Mahmood).

4. Results Chapter 2: Nicotine

4.1. Investigating cholinergic pathways in *L. variegatus*

As nicotine exerts its effects through nicotinic acetylcholine receptors, using sequences of all nAChR subunits and M1-M5 mAChRs, the *Homo sapiens* alignment for each subunit was compared to the alignments for each of the other species, *Rattus*, *Mus*, *Xenopus* and *Caenorhabditis* (Appendix 7-22). Percentage similarities were recorded in Table 4.1., highlighting the conservation of each subunit from human to *Caenorhabditis*.

Table 4.1. shows that mAChRs M1-3 and M5 are conserved down to *Caenorhabditis* however M4 is not. M1 has the most conserved protein sequence of the mAChRs for all species except for *Xenopus*, showing the highest level of conservation in *Mus* at 98.91%, *Rattus* at 98.70% and *C. elegans* at 50.00%. Within *Xenopus,* M2 exhibits the highest level of conservation at 81.36% compared to the other mAChRs. Whist M5 has the lowest conserved protein sequence for *Rattus* and *Mus*, it is the second highest conserved sequence in *C. elegans*.

Limited data was available for the nAChR subunits with only nAChR β2 and β4 sequences observed across all four species down to *C. elegans* (Table 4.1.). NAChR β2 shows the highest level of protein conservation in *Rattus* at 94.72%, *Mus* at 93.63% and *Xenopus* at 85.29% whereas nAChR β4 shows the highest conservation in *C. elegans* at 45.32%. *C. elegans* express a nAChR α7 homolog which shows a conservation level of 45.31% compared to the *Homo sapiens* nAChR α7 however other species data was not available for this receptor. Of all four species, *Mus* expresses the highest number of nAChR subunits with conservation levels of all expressed protein sequences being >80% (Table 4.1.).

Table 4.1. Conservation of mAChRs and nAChR subunits from human to genus. Alignments of all mAChRs and all known nAChR receptor subunits were run for *Homo sapiens*, *Rattus*, *Mus*, *Xenopus* and *Caenorhabditis.* Using BLAST, the percentage similarities between human alignments and other species were compared to calculate the conservation of the receptors and subunits across species.

Human Protein	Conservation of Human to Genus			
	Rattus	*Mus*	*Xenopus*	*Caenorhabditis*
Muscarinic acetylcholine receptor M1	98.70%	98.91%	56.55%	50.00%
Muscarinic acetylcholine receptor M2	96.27%	96.27%	81.36%	37.62%
Muscarinic acetylcholine receptor M3	91.69%	91.69%	62.90%	38.32%
Muscarinic acetylcholine receptor M4	95.82%	95.83%	74.15%	-
Muscarinic acetylcholine receptor M5	88.72%	88.72%	72.34%	49.52%
Nicotinic Cholinergic Receptor α1	-	90.43%	74.11%	-
Nicotinic Cholinergic Receptor α2	-	81.12%	-	-
Cholinergic Receptor Nicotinic α3	94.52%	92.89%	-	-
Cholinergic Receptor Nicotinic α4	92.29%	92.48%	71.40%	-
Cholinergic Receptor Nicotinic α5	-	89.91%	-	-
Cholinergic Receptor Nicotinic α6	-	85.02%	-	-
Cholinergic Receptor Nicotinic α7	-	-	-	45.31%
Cholinergic Receptor Nicotinic α8	-	-	-	-
Cholinergic Receptor Nicotinic α9	91.02%	-	-	-
Cholinergic Receptor Nicotinic α10	-	-	-	-
Cholinergic Receptor Nicotinic β1	-	-	-	-
Cholinergic Receptor Nicotinic β2	94.72%	93.63%	85.29%	39.10%
Cholinergic Receptor Nicotinic β3	-	-	-	-
Cholinergic Receptor Nicotinic β4	84.24%	84.91%	69.07%	45.32%
Cholinergic Receptor Nicotinic δ	-	-	-	-
Cholinergic Receptor Nicotinic γ	89.79%	89.79%	-	-
Cholinergic Receptor Nicotinic ε	-	-	-	-

As it has been previously determined that *L. variegatus* display a reduced behavioural response to nicotine (Appendix Figure 23), this has suggested that *L. variegatus* may express a cholinergic system through which nicotine exerts its effects. To begin to determine the presence of a cholinergic system in *L. variegatus*, the endogenous concentration of acetylcholine as well as acetylcholinesterase activity in *L. variegatus* was quantified.

Figure 4.1. A highlights that we observed the presence of endogenous acetylcholine in *L. variegatus* at an average concentration of 55.60 ng/μL. A confirmed presence of endogenous acetylcholine in *L. variegatus* would also suggest that there would be endogenous acetylcholinesterase activity which was confirmed as shown in Figure 4.1. B at an average of 1458.63 mU/mL.

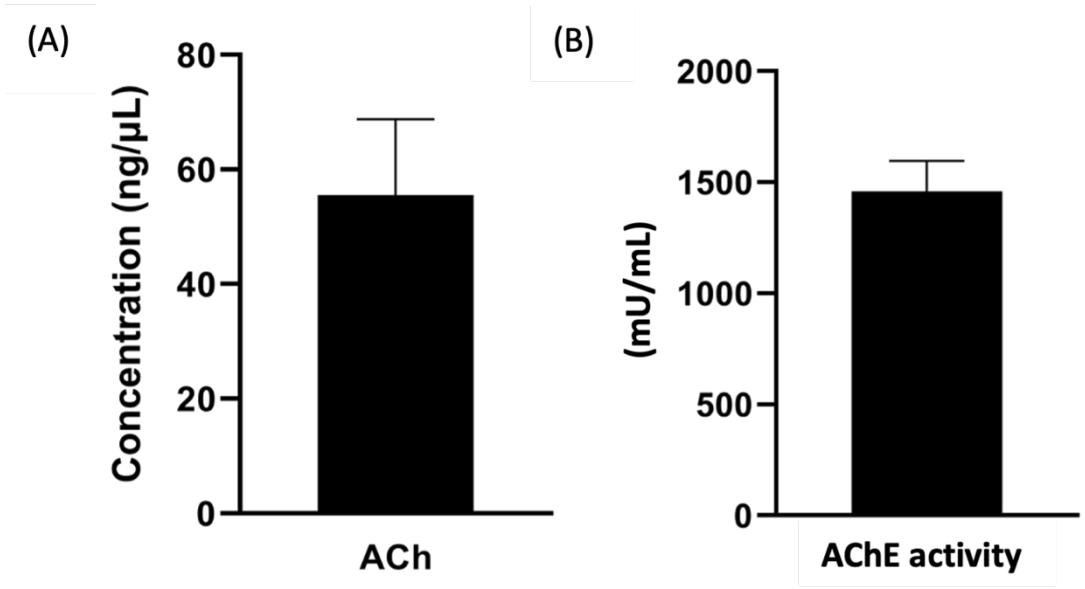


Figure 4.1. Quantifying the concentration of endogenous acetylcholine (ACh) and acetylcholinesterase (AChE) activity in *Lumbriculus variegatus*. Homogenised *L. variegatus* (using an Argos Tissue Homogeniser) were centrifuged at 16.1G for 15 minutes at 4°C. Manufacturer's guidelines were used on the supernatants to measure the concentrations of ACh or AChE activity. **(A)** Endogenous acetylcholine (Ach) was measured using a Choline/Acetylcholine Quantification Kit (MAK056, Sigma, St Louis, MO). Values are the mean ± SEM, n=6 experimental replicates measured in duplicate with twenty *L. variegatus* per sample. **(B)** Acetylcholinesterase (AChE) activity was measured using an Acetylcholinesterase Assay Kit (ab138871, Abcam, Cambridge, MA). Values are the mean ± SEM, n=3 experimental replicates measured in triplicate with twenty *L. variegatus* per sample (Data was generated and analysed in collaboration with Aidan Seeley).

Similarly, to ethanol, as short-term effects of nicotine exposure had been observed *in vivo*, Western blotting was completed to observe the short-term effects of nicotine *in vitro,* using the same markers for toxicity: Hsp70, Hsp60 and cytochrome C.

Vehicle-treated and 0.1 mM nicotine-treated *L. variegatus* protein samples were run through an SDS-PAGE gel and transferred to a membrane. Membranes were probed with anti-HSP1A1, anti-HSPD1 and anti-Cytochrome C and imaged (Figure 4.2. A).

We observed that like ethanol, Hsp60 and cytochrome C expression between the vehicle-treated and nicotine-treated *L. variegatus* samples showed no significant change (Figure 4.2. C & D). However, unlike what we observed with ethanol, there was also no significant change observed in the expression of Hsp70 between the two *L. variegatus* samples (Figure 4.2. B).

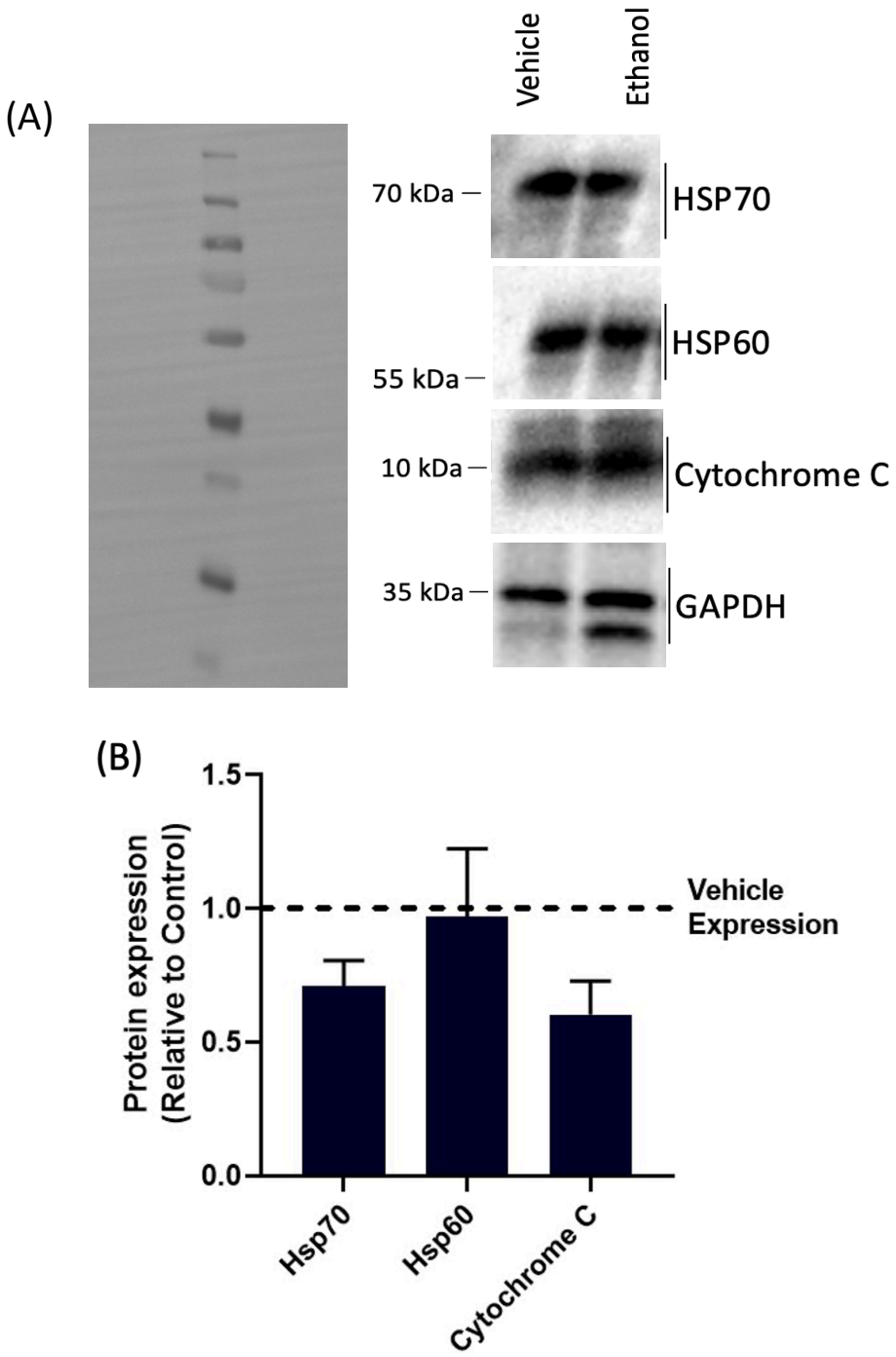


Figure 4.2. Western Blotting of Hsp60, Hsp70 and Cytochrome C expression in vehicle-treated and nicotine-treated *Lumbriculus variegatus*. Prior to protein extraction, *L. variegatus* were treated in either artificial pondwater or 0.1 mM nicotine for 10 minutes. *L. variegatus* protein samples were prepared and run through 8 – 12% SDS-PAGE gel. **(A)** Western blotting was completed using anti-HSPA1A, anti-HSPD1 and anti-Cytochrome C antibodies. GAPDH was used as a loading control. **(B)** Densitometry analysis quantified the Western blot of protein expression in *L. variegatus* exposed to 0.1 mM nicotine relative to protein expression in control *L. variegatus*, "Vehicle", exposed to artificial pondwater. Protein expression was normalised to GAPDH loading control. Error bars represent the standard error of the mean from three individual experiments. Statistical analysis was carried out using a one-way paired Student's t-test, *$p<0.05$ (data was generated in collaboration with Julanta Carriere and analysed by Romessa Mahmood).

To expand our understanding of the receptor pathways expressed in *L. variegatus*, we investigated the presence of a cholinergic receptor system in *L. variegatus* by administering increasing concentrations of mecamylamine, a nAChR antagonist, to *L. variegatus*.

Figure 4.3. A & B shows that administering increasing concentrations of mecamylamine results in no significant change in body reversal and helical swimming movement ($p>0.05$). This is also seen 10-mins following mecamylamine removal ($p>0.05$) however at 24 hours following mecamylamine removal, *L. variegatus* exposed to $\geq$50 μM had significantly reduced body and reversal and helical swimming ($p=0.0027$) (Figure 4.3. C & D).

Figure 4.3. E shows that a lack of significant difference is also seen in *L. variegatus* free locomotory movement following mecamylamine administration. This is also seen 10-mins and 24 hours following mecamylamine removal where movement stays at baseline levels (Figure 4.3. G).

The LOAEL for mecamylamine was established to be 50 μM. The NOAEL for mecamylamine was established to be 10 μM.

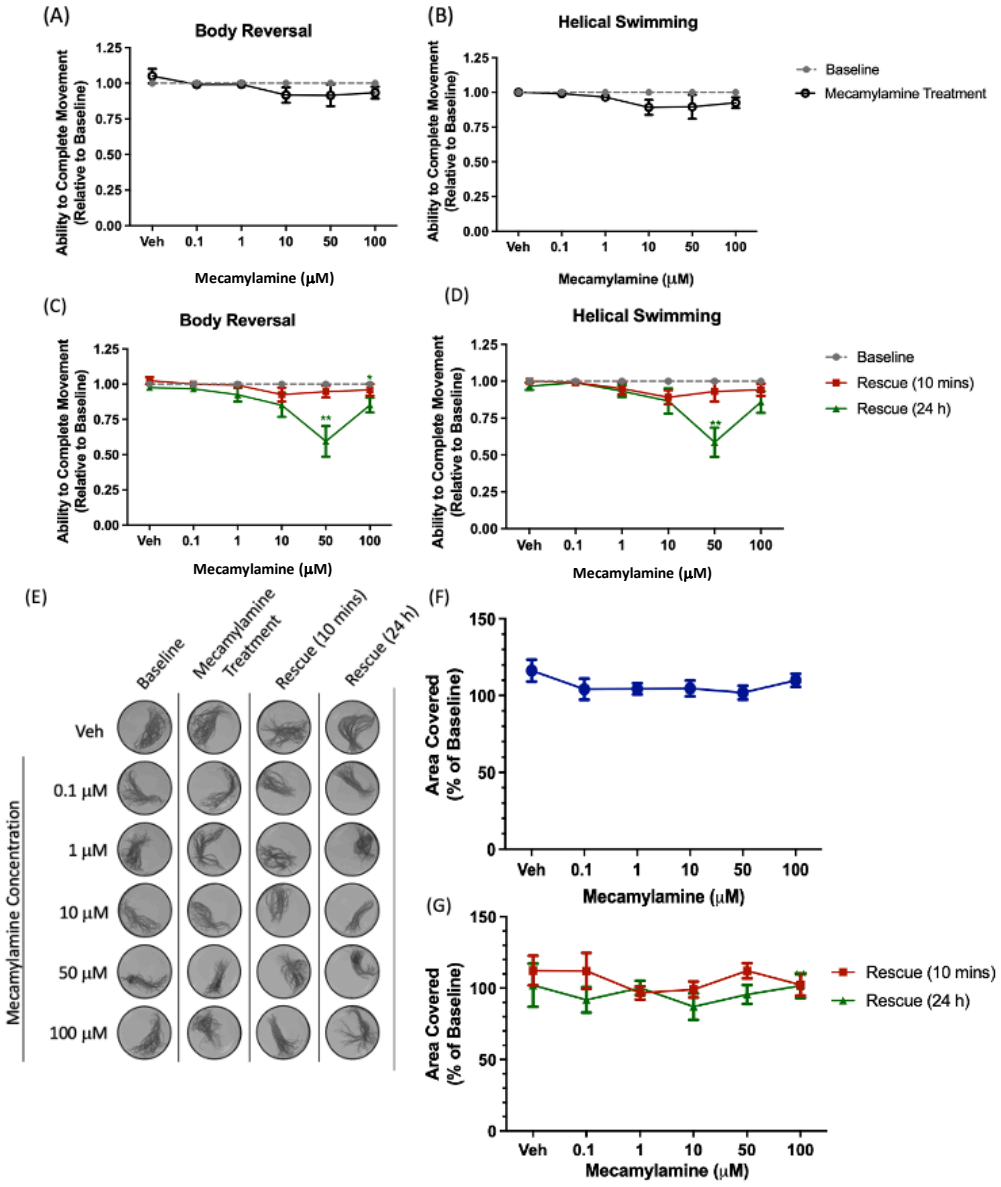


Figure 4.3. The effect of mecamylamine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of mecamylamine (0 – 100 μM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Mecamylamine was then removed 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 mecamylamine on free locomotion was measured before mecamylamine exposure (Baseline), after 10 minutes of exposure to 0 – 100 μM mecamylamine, 10 minutes after mecamylamine removal (Rescue (10 mins)) and 24 hours after mecamylamine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** mecamylamine treatment and **(G)** removal of mecamylamine for 10 minutes and 24 hours are expressed as a percentage of the area covered at baseline, n=8 experimental replicates for each concentration. Veh: artificial pondwater. p*<0.05, p**<0.01. (Data was generated in collaboration with Ermando Canga and Vedika Vyas).

Tubocurarine is another nicotinic receptor antagonist.

Figure 4.4. A & B highlights that 10-mins exposure to tubocurarine results in a significant decrease in *L. variegatus* body reversal and helical swimming at 25 μM ($p=0.0156$), 50 μM ($p=0.0078$) and 100 μM ($p=0.0078$). These effects are reversed 10-mins and 24 hours following tubocurarine removal, where body reversal and helical swimming return to baseline levels ($p>0.05$) (Figure 4.4. C & D).

Figure 4.4. F shows that following 10-mins exposure to increasing concentrations of tubocurarine, no significant change in free locomotory movement of *L. variegatus* is observed ($p>0.05$). This is also seen 10-mins and 24 hours following tubocurarine removal where movement stays at baseline levels (Figure 4.4. G).

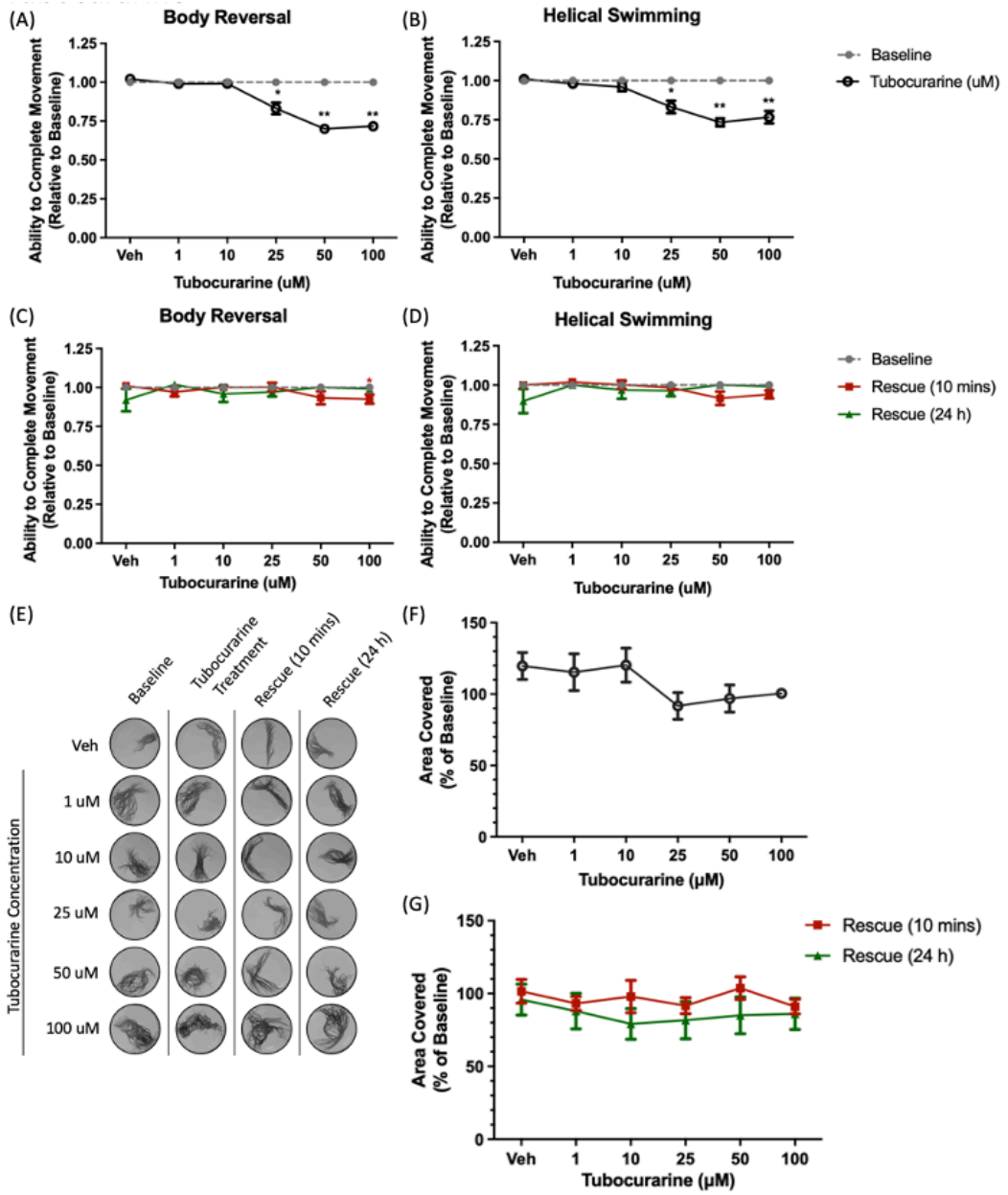


Figure 4.4. The effect of tubocurarine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of tubocurarine (0 – 100 µM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Tubocurarine was then removed 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 tubocurarine on free locomotion was measured before tubocurarine exposure (Baseline), after 10 minutes of exposure to 0 – 100 µM tubocurarine, 10 minutes after tubocurarine removal (Rescue (10 mins)) and 24 hours after tubocurarine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F)** tubocurarine treatment and **(G)** removal of tubocurarine for 10 minutes and 24 hours are the mean, n=7 experimental replicates for each concentration. Veh: artificial pondwater. *p<0.05, **p<0.01 (Data was generated in collaboration with Ermando Canga and Vedika Vyas and analysed by Romessa Mahmood).

To further investigate the cholinergic receptor pathways in *L. variegatus*, *L. variegatus* were pre-treated with either artificial pond water, 100 μM mecamylamine or 10 μM tubocurarine and then treated with 0.1 mM nicotine.

Figure 4.5. B shows that nicotine alone caused a significant reduction in *L. variegatus* free locomotory movement by 65.82%±3.82% ($p<0.0001$). This behavioural response was significantly lessened when *L. variegatus* were pre-treated with mecamylamine, which caused movement to reduce by 41.73%±9.47% ($p=0.0026$) following 10-mins exposure meaning that movement increased by 24.09%. 10-mins following nicotine removal, there was no significant difference in the decrease of free locomotory movement between pre-treatment of *L. variegatus* with mecamylamine and nicotine alone ($p<0.05$). Free locomotory movement returned to baseline levels for both pre-treatment of mecamylamine and nicotine alone 24 hours following nicotine removal.

The reduction in free locomotory movement induced by nicotine was significantly potentiated when *L. variegatus* were pre-treated with 10 μM tubocurarine, compared to nicotine exposure alone (Figure 4.5. B). Whereas nicotine alone caused movement to reduce by 65.82%±3.82% ($p<0.001$), pre-treatment with tubocurarine caused movement to reduce by 91.04%± 0.63% ($p<0.0001$) following 10-mins exposure suggesting that movement was further reduced by 25.22%. This significant difference in the reduction of free locomotory movement of pre-treatment with tubocurarine in comparison to nicotine alone persisted 10-mins following nicotine removal ($p<0.0001$). Free locomotory movement returned to baseline levels for both pre-treatment of tubocurarine and nicotine alone 24 hours following nicotine removal.

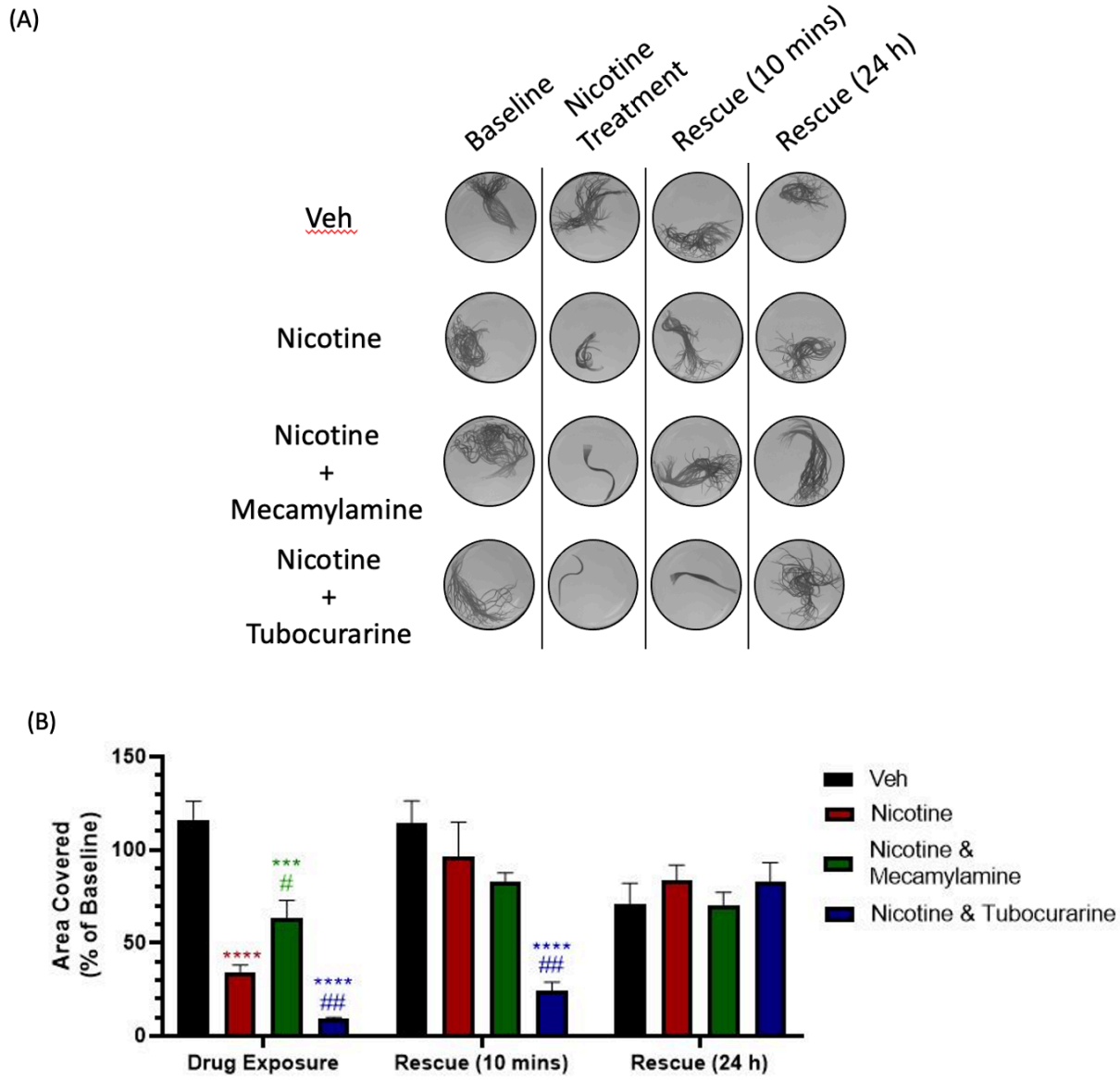


Figure 4.5. Comparison of the effects of nicotine alone and pre-treatment with mecamylamine or tubocurarine on *Lumbriculus variegatus* behaviour. *L. variegatus* were pre-treated with either artificial pondwater 100 mM mecamylamine or 10 mM tubocurarine for 10 minutes. Following removal of pre-treatment, *L. variegatus* were exposed to 0.1 mM nicotine. (A) The effect of nicotine on free locomotion was measured before nicotine exposure (Baseline), after 10 minutes of exposure to 0.1 mM nicotine, 10 minutes after nicotine removal (Rescue (10 mins)) and 24 hours after nicotine removal (Rescue (24 h)). (B) Quantification of the area covered by *L. variegatus* following nicotine treatment and removal of nicotine for 10 minutes and 24 hours are the mean, n=6 experimental replicates for each concentration. Veh: artificial pondwater. Statistical differences were measured by two-way ANOVA compared to the vehicle or 10 minutes nicotine exposure, * shows statistical significance for drug exposure relative to vehicle, # shows the statistical significance for drug exposure relative to nicotine, #p<0.05, ##p<0.01, ***p<0.001, ****p<0.0001 (Data was generated in collaboration with Ermando Canga, Vedika Vyas and Julanta Carriere).

To observe whether the order of administration of the antagonist and nicotine would affect *L. variegatus*' response to nicotine, 100 μM mecamylamine or 10 μM tubocurarine were co-administered with 0.1 mM nicotine.

Figure 4.6. B shows that following 10-mins exposure to either nicotine or antagonist + nicotine, co-administration does not result in any significant difference in *L. variegatus*' response to nicotine. Nicotine administered alone resulted in a significant decrease of free locomotory movement by 66.20%±6.04% ($p<0.0001$), co-administration with mecamylamine resulted in a significant decrease of free locomotory movement by 65.51%±7.97% ($p<0.0001$) and co-administration with tubocurarine resulted in a significant decrease of free locomotory movement by 77.50%±4.62% ($p<0.0001$). This significant decrease in movement was reversed for all three conditions 10-mins and 24 hours following nicotine removal ($p>0.05$).

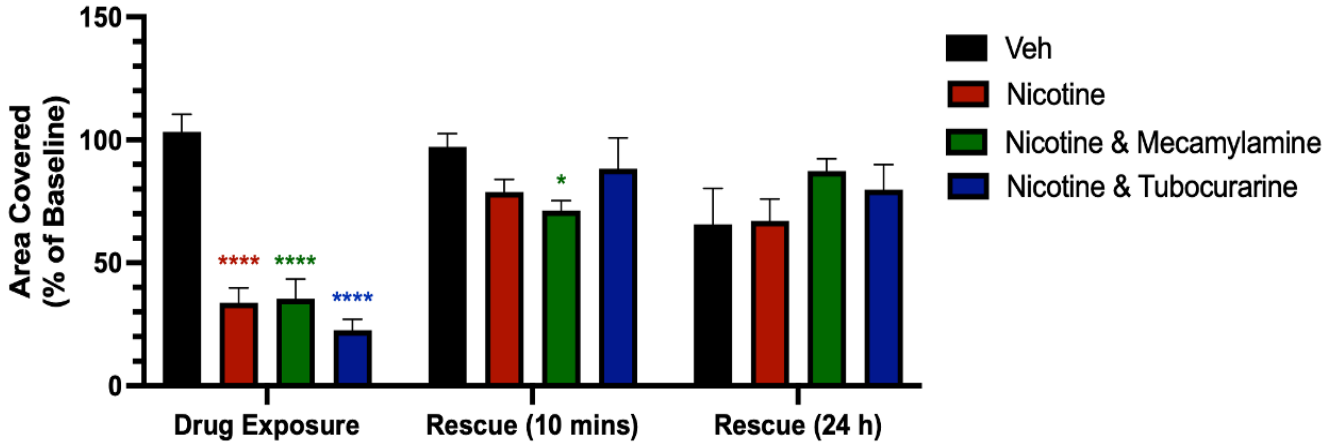


Figure 4.6. Comparison of the effects of nicotine alone and co-administration with mecamylamine or tubocurarine on *Lumbriculus variegatus* behaviour. *L. variegatus* were treated with either artificial pondwater or one of the following nicotinic antagonists which had been spiked with 0.1 mM nicotine: 100 μM mecamylamine or 10 μM tubocurarine for 10 minutes. The effect of nicotine on free locomotion was measured before nicotine exposure (Baseline), after 10 minutes of exposure to 0.1 mM nicotine, 10 minutes after nicotine removal (Rescue (10 mins)) and 24 hours after nicotine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following nicotine treatment and removal of nicotine for 10 minutes and 24 hours are the mean, n=6 experimental replicates for each concentration. Veh: artificial pondwater. Statistical differences were measured by two-way ANOVA compared to the vehicle, *$p<0.05$, ****$p<0.0001$ (data was generated in collaboration with Ermando Canga, Vedika Vyas and Julanta Carriere).

5. Discussion

Vertebrate animal models provide an essential tool to explore drugs of abuse and addiction in pre-clinical stages as their use requires fewer ethical restrictions compared to clinical trials and still allows for the replication of drug-taking behaviour (Müller, 2018). Nevertheless, due to the efforts of Russell & Burch (1960), as well as laws such as ASPA (Animals (Scientific Procedures) Act 1986, 1986), and ethical and moral implications of animals in research, there is a need to reduce the number of vertebrate models being used in research. Whilst there are current established invertebrate models used within pharmacological toxicity studies, including the nematode *C. elegans* (Engleman et al., 2016) and fruit fly *D. melanogaster* (Kaun et al., 2012), both of these models have their limitations due to their small sizes. *L. variegatus* has been widely established as a model for aquatic toxicity (O'Gara et al., 2004; Phipps et al., 1993; Wallin et al., 2018) however its use as a model for pharmacological toxicity, including drugs of abuse, has been less extensively explored (Seeley et al., 2021). *L. variegatus* are comparatively larger than *C. elegans* and *D. melanogaster* at 5 – 8 cm (Seeley et al., 2021), which have average lengths of 1 mm (Andrews, 2019) and 3 mm (Shimazaki et al., 2022) respectively and therefore, requiring microscopy for behavioural imaging and analysis (Breimann et al., 2019; Pende et al., 2018). Due to the larger size of *L. variegatus*, Seeley et al., (2021) were able to optimise the *L. variegatus* behavioural stereotypical movement and free locomotory assays; these assays were used within this study to administer ethanol and nicotine, as well as neurotransmitters and their agonists and antagonists to the worms and allowed their behaviour to be observed and quantified without the need of any specialist machinery.

5.1. Ethanol

Exposing *L. variegatus* to ethanol has been previously shown to induce a dose-dependent reduction in both stereotypical movement (Appendix Figure 1 A & B) and free locomotory movement (Appendix Figure 1 F) at concentrations ≥250 mM (Bellamy, 2023), with this study demonstrating the response starting at 2 minutes during a 10-minute ethanol exposure period (Figure 3.1.). Existing data highlights that administration of similar concentrations of ethanol

inhibits movement in *C. elegans*, where 100 – 500 mM ethanol inhibited thrashing movement at 1 minute (Mitchell et al., 2007). When exposed to 500 mM ethanol, *L. variegatus* were observed to reach maximum inhibition of free locomotory movement at 6 minutes (Figure 3.1.) whereas Mitchell et al., (2007) demonstrated that, when exposed to the same ethanol concentration, the maximum inhibition of thrashing of *C. elegans* was reached at a faster timepoint of 3 minutes. In comparison to *L. variegatus* and *C. elegans*, whose behaviour decrease when exposed to ethanol, *D. melanogaster* demonstrate a biphasic response to ethanol; during the first 10 minutes of exposure to ethanol, flies exhibit an increase in locomotory behaviour which then reduces as the time exposed to ethanol increases (Bainton et al., 2000). This is thought to be more representative of the behavioural response to ethanol observed in humans, where initial exposure to ethanol results in euphoria and hyperactivity and as the BAC rises, individuals exhibit motor impairment (Chvilicek et al., 2020). Following a 10-minute recovery period where *L. variegatus* were removed from ethanol, both stereotypical behaviours (Appendix Figure 1 C & D) and locomotory behaviours (Appendix Figure 1 G) returned to baseline levels which continued after 24 hours. As *C. elegans* demonstrated full recovery in movement 2 minutes following ethanol removal (Mitchell et al., 2007), it would be beneficial to observe at what timepoint *L. variegatus* recover during the 10-minute recovery period. Administering lower concentrations of ≤50 mM ethanol, resulted in no change in *L. variegatus* stereotypical and free locomotory movement (Figure 3.2.) however this does not support the findings of existing studies in other invertebrates. For example, in lower concentrations in *D. melanogaster*, 15 minutes exposure to 15 mM ethanol vapour resulted in a reduction in locomotory behaviour (Bainton et al., 2000). Reduced locomotory movement was also observed at concentrations of 20 and 30 mM ethanol when administered to *C. elegans* (Davies et al., 2003). These findings in invertebrate models contradict what has been observed in the CNS of vertebrate models, where exposure of rat neurons to 10 - 40 mM ethanol has been shown to decrease the inhibitory response caused by $GABA_A$-receptor mediated currents (Xiao & Ye, 2008).

As we established that there was an *in vivo* behavioural response to acute ethanol exposure, we aimed to explore the *in vitro* response via the expression of heat shock proteins (Hsp), Hsp70 and Hsp60, both of which have been shown to increase in expression following acute ethanol exposure in *C. elegans* (Kwon et al., 2004) as well as Cytochrome C, which also

increases following chronic ethanol consumption (Graw et al., 2015). As shown in Figure 3.3. B, ethanol-treated *L. variegatus* showed a significantly higher expression of Hsp70 than the vehicle-treated worms. This ethanol-induced increase in Hsp70 expression is observed across vertebrate and invertebrate models: Hsp70 levels have been shown to increase in both rat brain and liver following 7-days of alcohol exposure (Calabrese et al., 2000) and in honeybee brain tissue following a 4h 5% (equivalent to 1,085 mM) ethanol administration period (Hranitz et al., 2010). Our results suggest that ethanol-induced oxidative stress occurs within *L. variegatus*. It is interesting to note that whilst our highest concentration of 500 mM ethanol induced a stress response in *L. variegatus*, higher concentrations of 10% ethanol did not exhibit this response in honeybees (Hranitz et al., 2010). Therefore, it would be interesting to note whether lower concentrations of ethanol, such as 250 mM which induces a decrease in the *in vivo* response, would induce an increase in the expression of Hsp70 and whether this expression is significantly different to what is observed with 500 mM. Figure 3.3. C & D show that ethanol treatment did not induce an increase in the expression of Hsp60 and cytochrome c. Whilst Hsp60 production is induced during oxidative stress, there are currently conflicting findings linking this to ethanol administration. Whereas Rakonczay et al., (2003) found that there was no induction of Hsp60 in ethanol-administered mice, Malik et al., (2013) observed that Hsp60 in exosomes released by cardiac myocytes were greatly increased. To date, there is no data available observing this in invertebrate models.

5.2. Ethanol tolerance in *L. variegatus*

Establishing that an acute, short exposure to ethanol resulted in reduced *L. variegatus* behaviour, we aimed to observe the effects of longer exposure to ethanol and whether *L. variegatus* developed tolerance to ethanol. Tolerance to ethanol can be described as either acute or chronic. Exposing *L. variegatus* to 500 mM ethanol over a 210-minute time period, we aimed to observe whether *L. variegatus* develop AFT. Figure 3.4. highlights that after the initial 10-minute exposure period to which all other timepoints were compared, at the 150- and 210-minute timepoints, free locomotory movement increased, therefore suggesting that *L. variegatus* are able to develop AFT. This response may be able to be explained by the Mellanby effect model, where during the first stages of drug exposure, there is a rapid increase to experiencing the peak drug effect, as seen at 50-mins in *L. variegatus*. Although the rate of

ethanol excretion in *L. variegatus* is not known, the significant increase in *L. variegatus* behaviour at 150- and 210-mins follows the descending limb seen in the Mellanby effect, where it takes longer for both the BAC and the drug effect to decrease (Mellanby, 1919). As it been observed that L. variegatus follow the behaviour pattern graphed by Mellanby (1919) during the development of AFT, it would be interesting to observe how the BAC of L. variegatus also changes at each time point that was measured during AFT. Other invertebrate models, including *C. elegans* (Alaimo et al., 2012) and *D. melanogaster* (Scholz et al., 2000) have also been shown to develop AFT at ethanol concentrations of 400 mM and 40 mM respectively. When observing AFT, we also observed that following the initial 10-minute exposure, *L. variegatus* free locomotory movement significantly decreased further at the 30- and 50-minute timepoints. This differs to *C. elegans* where (Alaimo et al., 2012) demonstrated that at 50 minutes, *C. elegans* had developed AFT and exhibited a significant increase in locomotion. Chronic tolerance is defined as tolerance that develops over multiple sessions of ethanol consumption which occurs over a timespan of multiple days and can include both continuous and intermittent use (Pietrzykowski & Treistman, 2008). Before we observed the effects of chronic ethanol consumption on *L. variegatus*, we aimed to establish the median lethal dose (LD_{50}) of ethanol on *L. variegatus* when exposed to increasing concentrations up to 500 mM ethanol. Figure 3.5. shows that we did not observe any long-term toxicity of acute ethanol exposure on *L. variegatus* behaviour at concentrations $\leq$500 mM ethanol. This led us to culturing *L. variegatus* in 100 mM ethanol for $\geq$21 days so that we could observe whether chronic consumption of ethanol led to *L. variegatus* developing chronic tolerance by observing any changes in the behavioural response to 10-minute ethanol exposure. 100 mM ethanol was used for the culture as this was the established NOAEL of ethanol in *L. variegatus*, where 250 mM and 500 mM induced significant inhibition of movement. We determined that chronic consumption of ethanol did not result in *L. variegatus* developing chronic tolerance as Figure 3.6. B-D highlights that there was no significant change in the behavioural response to increasing concentrations of ethanol between ethanol-naïve and ethanol-cultured *L. variegatus*. Whilst a direct comparison of ethanol-naïve and ethanol-cultured models is not available, increasing exposure to ethanol vapour results in *Apis mellifera* developing chronic tolerance to ethanol (Miler et al., 2018). Similar results with prolonged exposure to low concentrations of ethanol vapour were seen in *D. melanogaster*, where the recovery time of flies pre-exposed to ethanol was faster than the recovery times of the control group (Berger

et al., 2004). We also calculated the changes in *L. variegatus* body size due to chronic ethanol treatment and whether this affected baseline movement. Figure 3.7. A demonstrates that chronic ethanol exposure results in a significant increase in *L. variegatus* body size. This contrasts with existing invertebrate models such as C. elegans, where chronic ethanol exposure reduced *C. elegans*' length of body which was thought to be due to a reduced intake of food compared to control worms (Davis et al., 2008). The same study also demonstrated, however, that chronic ethanol exposure at 100 mM and 200 mM over the lifespan of *C. elegans* resulted in the longer worms surviving over the shorter worms which was suggested to be due to the shorter worms not being able to endure the damage caused as a result of the ethanol exposure (Davis et al., 2008). It could be theorised that the average body length of *L. variegatus* increases when chronically exposed to ethanol due to inhibition of fragmentation, the process by which the worms asexually reproduce (Zattara & Bely, 2016) however more research would be required in worms to explore the effect of toxic substances such as ethanol on fragmentation.

Whilst we did not observe the impact of chronic ethanol exposure on the lifespan of *L. variegatus*, *C. elegans* were shown to have a reduced lifespan (Davis et al., 2008) and this would therefore be beneficial to observe in *L. variegatus*. Another beneficial observation to make would be whether removing *L. variegatus* from the chronic ethanol environment would allow growth to resume as normal. When removing matured *C. elegans* from chronic ethanol exposure, ethanol treated worms had grown to the same size as the control worms (Davis et al., 2008).

5.3. Fatty acid expression

Observing the physiological changes that chronic exposure to ethanol can cause to *L. variegatus* body size, we wanted to observe whether there were any changes *in vitro*. Chronic alcohol consumption leading to an accumulation of fatty acids, known as fatty liver, has been well characterised in current literature in humans (Sozio & Crabb, 2008; You & Arteel, 2019) and in vertebrate models such as rats (Ojeda et al., 2008) and mice (Wei et al., 2013). This has not, however, been as extensively studied in invertebrate models and therefore we aimed to

observe whether *L. variegatus* expressed fatty acids and if this expression changed when chronically exposed to ethanol.

Stearic acid is a naturally occurring saturated long-chain fatty acid in humans and animals, shown to regulate the structure and function of mitochondria (Senyilmaz-Tiebe et al., 2018). When consumed, increased levels of stearic acid are also shown to reduce the risk of cardiovascular disease (Kris-Etherton et al., 2005) and cancer (Cross et al., 2014; Kühn et al., 2016). Palmitic acid is another long-chain fatty acid, synthesised in the body from other fatty acids as well as carbohydrates and amino acids (Ortega & Campos, 2021). Unlike stearic acid, increased levels of palmitic acid are shown to increase the risk of atherosclerosis (Siri-Tarino et al., 2010) and cancer (Mancini et al., 2015).

Table 3.1. shows that in both the ethanol naïve and ethanol cultured *L. variegatus* samples, the expression of stearic acid and palmitic acid were observed but there was no significant change in the levels of expression after ethanol exposure. Previously, it has been shown that chronic consumption of alcohol can result in reduced levels of both stearic acid and palmitic acid in the human intestine (Chen et al., 2014). In the same study, it was shown that whilst stearic and palmitic acid did not directly protect the gut from alcohol-induced damage, *Lactobacillus* species, responsible in the gut microbiota for barrier function in the intestine, would metabolise these long chain fatty acids to help promote their function. This has been supported in more recent studies, where Nie et al., (2022) demonstrated that stearic acid in the gut may also help to regulate gut microbiota and therefore prevent liver damage caused by excess alcohol consumption. In *C. elegans* larvae, whilst the protective factor of these acids following chronic ethanol consumption is unknown, following a 2 to 3 day incubation in low concentration ethanol, the ability to convert low concentration ethanol into fatty acids to allow the larvae to live for longer and store fuel, has been demonstrated (Castro et al., 2012). It would be beneficial to explore further whether *L. variegatus* demonstrate a similar ability.

Table 3.1. also shows that whilst not seen in the ethanol naïve *L. variegatus* samples, the presence of oleic acids was observed in the ethanol cultured samples. Oleic acid is a long-chain fatty acid synthesised by stearoyl-CoA desaturase 1 (SCD1), which uses stearic acid as its substrate. These results suggest an active role of desaturases within *L. variegatus*. In mice,

oleic acid, similar to stearic acid, has also been demonstrated to reduce long-term ethanol-induced liver damage (Zirnheld et al., 2019). In human patients suffering with alcohol dependence, Teubert et al., (2013) observed that the serum concentration of oleic acid was raised compared to patients who do not have alcohol dependence. Oleic acid's role for long-term ethanol exposure has not been extensively explored in invertebrates.

As there are limited studies available looking at the levels of these fatty acids in invertebrate models following chronic ethanol consumption, it would be beneficial to further explore.

5.4. GABAergic receptor pathway

As the GABAergic receptor pathway has been a proposed pathway for the mechanism of action for ethanol (Kumar et al., 2009), our next set of experiments involved exploring the presence of a GABA receptor pathway within *L. variegatus*. By determining the percentage similarities of the protein sequences of $GABA_A$ receptor subunits α1, β2 and γ2 (Pirker et al., 2000) and $GABA_B$ receptor subunits 1 and 2 (Jones et al., 1998) in the genera *Homo sapiens*, *Rattus*, *Mus*, *Xenopus* and *Caenorhabditis*, we were able to look at the conservation of these subunits from *Homo sapiens* in other animal models, starting from the genus *Rattus* down to *Caenorhabditis*. However, as *L. variegatus* is not genotyped, the protein sequences are not available and therefore unable to be included in the analysis.

Table 3.2. shows that whilst the $GABA_A$ receptor subunits were conserved down to *Xenopus*, with the exception of β2 which only showed conservation in *Rattus* and *Mus*, they were not conserved down to *Caenorhabditis*. Invertebrates, like *C. elegans*, have been demonstrated to have their own unique GABA receptors however these show pharmacological differences to vertebrate GABA receptors (Dent, 2006) despite the similarities in structure with five subunits making up a transmembrane ion channel (McGonigle & Lummis, 2009). This is because they are "Resistance to Dieldrin" (RDL) receptors (Dent, 2006), which were first identified in *Drosophila* (Ffrench-Constant et al., 1991). *C. elegans* express a ligand-gated calcium ion channel GABA receptor that is responsible for GABA-mediated muscular inhibitory neurotransmission in nematodes (McIntire et al., 1993). This GABA receptor is encoded by the *unc-49* gene and can be homomeric, made up of the UNC-49B subunit, or heteromeric, made

up of the UNC-49A, UNC-49B and UNC-49C subunits (Bamber et al., 1999). Whilst various $GABA_A$ subunits, such as α4, β3 and γ2, have been suggested to increase the receptor sensitivity to ethanol (Mihic et al., 1994; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), there have not been any studies able to confirm the exact subunits that ethanol interacts with to exert its effects.

Table 3.2. also shows that whilst both $GABA_B$ receptor subunits are conserved down to *Caenorhabditis*, there is no data available for the expression of $GABA_B$ receptor subunit 1 ($GABA_{B(1)}$) in *Xenopus*. In *C. elegans*, whilst it is established that the $GABA_B$ receptors are responsible for locomotory movement when activated by GABA (Dittman & Kaplan, 2008), there is no evidence as to the role of $GABA_B$ in the behavioural response to ethanol. In *Drosophila*, however, $GABA_B$ receptors have been demonstrated to provide a protective role to the effects of ethanol by reducing the acute ethanol-induced behavioural responses seen and reducing the development of alcohol tolerance (Dzitoyeva et al., 2003). $GABA_{B(1)}$ knockout mice demonstrate a higher consumption of alcohol and show a preference to alcohol over water, as well as reaching a higher alcohol blood concentration compared to wild-type mice (Floris et al., 2022) highlighting that the $GABA_B$ receptor pathway plays a more regulatory role in ethanol consumption, rather than mediating the effects of ethanol. In *C. elegans*, the $GABA_B$ receptors are responsible for the release of GABA and knowing that *C. elegans* express both GABA receptor pathways highlights the significance of the pathways in regard to their physiological functions and therefore the expression of these pathways in *L. variegatus* was explored.

5.5. GABA

To explore whether *L. variegatus* express a GABAergic system, Bellamy (2023) administered increasing concentrations of GABA to the worms (Appendix Figure 5). Body reversal and helical swimming movements were dose-dependently reduced, however, the same response was not observed for free locomotory movement, where no significant change in behaviour was observed. In the CNS of vertebrate models, GABAergic neurons make up 40% of inhibitory synapses through which GABA acts as an inhibitory neurotransmitter, preventing the firing of action potentials (Wu & Sun, 2015). Whilst this would provide an explanation as to the

stereotypical behavioural response of *L. variegatus* to GABA, it does not explain why there was no free locomotory response observed. Within *C. elegans*, <10% of the nervous system is made up of GABA neurons (Docherty et al., 1985) and GABA acts at neuromuscular junctions, exhibiting both inhibitory and excitatory functions in the muscle contraction of the worm depending on which neuron it is released from (Mclntire et al., 1993). Whilst GABA released from the AVL and DVB neurons promotes excitatory muscle contraction to allow *C. elegans* to defecate (Thomas, 1990), ventral cord D type neurons are inhibitory; when *C. elegans* are tapped on the nose, D-type neurons will release GABA which will relax the body muscle on one side whilst cholinergic-mediated ACh release will contract the body muscle on the opposite side (Mclntire et al., 1993), allowing waves to be propagated from the tail end to the head end resulting in a backwards movement (Croll, 1975). When tapping *L. variegatus* on its head, we observe a similar body reversal movement that is mediated by MGF via glutamatergic signals (Lybrand et al., 2020). Glutamate, an excitatory neurotransmitter (Zhou & Danbolt, 2014), is a precursor to the formation of GABA (Wong et al., 2003) and both neurotransmitters will often work together (Wong et al., 2003) meaning that they are both likely to be found together. Evidence has shown that when glutamate activates NMDA receptors, this can also lead to the release of GABA and therefore activation of GABA receptors (Lujan et al., 2005; Wen et al., 2022). From this, it could be hypothesised that following tactile stimulation of either the anterior or posterior end of *L. variegatus*, there is the activation of glutamatergic signalling which will also activate the release of endogenous GABA. When *L. variegatus* are then administered exogenous GABA, this increases the inhibitory neurotransmission of GABA, therefore reducing stereotypical movement. As free locomotory movement may not activate the release of endogenous GABA, this could be why there is no significant decrease in movement seen following the administration of GABA. This hypothesis is further strengthened as it has been demonstrated that when administered ivermectin, an antiparasitic, GABA receptors are inhibited in *C. elegans* (Hernando & Bouzat, 2014). Previous to this study, ivermectin had been administered to *L. variegatus* and it was observed that the nongiant interneuron pathways, responsible for movement such as free locomotion, were more sensitive to its effects compared to the giant interneuron pathways, responsible for stereotypical movement as previously mentioned (Ding et al., 2001). Another invertebrate model, although not a worm, *Procambarus clarkii* (*P. clarkii*), also known as crayfish, uses a

giant fibre neuronal pathway for its locomotory movements (Edwards et al., 1994) which has been observed to express a GABAergic pathway (Swierzbinski & Herberholz, 2018).

As we observed that *L. variegatus* displayed a behavioural response to GABA administration, suggesting that *L. variegatus* express a GABAergic system, we moved on to explore whether this was mediated by a $GABA_A$ receptor pathway, $GABA_B$ receptor pathway or both.

5.6. $GABA_A$ receptor pathway

To allow us to elucidate the expression of a $GABA_A$ receptor pathway, Bellamy (2023) also explored the behavioural responses to administering increasing concentrations of bicuculline, a $GABA_A$ receptor antagonist. Body reversal and helical swimming movements were dose-dependently reduced. The response in free locomotory movement to bicuculline differed as an increase in movement was seen at 5 μM bicuculline and a decrease in movement was only seen at the highest administered concentration of 250 μM. Despite the dose-dependent reduction seen in *L. variegatus* stereotypical movement, existing literature demonstrates that GABA receptors expressed in invertebrate models are not sensitive to bicuculline, including *C. elegans* (Bamber et al., 2003), *D. melanogaster* (Zhang et al., 1995), *Apis mellifera* (Palmer & Harvey, 2014) and *Manduca sexta* (a moth; Sattelle et al., (2003)). We wanted to further explore whether this could also be a possibility in *L. variegatus* by pre-administering the worms with 2.5 μM bicuculline and then exposing them to increasing concentrations of GABA; this would allow us to observe whether bicuculline inhibits the behavioural response seen to GABA. Pre-treatment of bicuculline did not result in any significant change to the dose-dependent reduction in body reversal and helical swimming seen following GABA administration alone (Figure 3.8. A & B). There was also no significant change in movement seen with the pre-administration of bicuculline in the free locomotory response to GABA administration (Figure 3.9.). A lack of sensitivity of invertebrate receptors to bicuculline has been reported to be due to their RDL characteristics (Zhang et al., 1995) however there is limited information available as to why this is. As bicuculline's main mechanism of action has been reported to occur through $GABA_A$ receptors (Yamazaki et al., 2020), this highlights the possibility of other pathways that bicuculline may act through. Although recent studies are

limited, older studies show that bicuculline can act through nAChRs (Hill et al., 1973; Demuro et al., 2001) and small conductance calcium activated potassium channels (Johnston, 2013).

As the $GABA_A$ receptor pathway has been a proposed mechanism of action for ethanol, we used the previous data presented by Bellamy (2023) and observed the behavioural response of *L. variegatus* to ethanol when pre-treated with either 1 mM GABA or 2.5 μM bicuculline. When ethanol allosterically binds to $GABA_A$ receptors, it results in the potentiation of a GABA current which in turn presents as ethanol having a sedative effect (Davies, 2003). Pre-treatment with GABA did not result in any change in free locomotory behaviour in response to ethanol (Figure 3.10. B).

In rodent models such as rats, bicuculline has been demonstrated to increase ethanol-seeking behaviour (Kemppainen et al., 2012). When pre-treated with bicuculline, at 100 mM ethanol there was a significant increase in *L. variegatus* free locomotory behaviour by 38.11% (Figure 3.10.) however at all other ethanol concentrations there was no significant change in behaviour observed. This increase may not be seen at the higher concentrations due to too much competition between ethanol and bicuculline to bind with the receptor.

5.7. $GABA_B$ receptor pathway

The $GABA_B$ receptor pathway has also been identified as playing a role in alcohol-related behaviours however this is in an opposite way to the $GABA_A$ receptor pathway as orthosteric binding of the $GABA_B$ receptor has been shown to control alcohol drinking and symptoms of alcohol withdrawal (Agabio & Colombo, 2014). As we were unable to establish the presence of a $GABA_A$ receptor pathway within *L. variegatus*, we aimed to explore the presence of a $GABA_B$ receptor pathway to explain the behavioural responses seen in *L. variegatus* to ethanol and GABA. Baclofen is the only $GABA_B$ receptor agonist available for human use (Durant et al., 2018) and has current therapeutic uses as a muscle relaxant and is also used off-label for the symptoms of alcohol withdrawal (de Beaurepaire et al., 2019). Administering increasing concentrations of baclofen to *L. variegatus* resulted in no significant change in body reversal and helical swimming (Figure 3.11. A & B) and in free locomotory movement (Figure 3.11. F). Similar results were observed in *D. melanogaster* (Mezler et al., 2001) and *Periplaneta*

americana (Bai & Sattelle, 1995) where baclofen administration failed to induce any response from the $GABA_B$ receptor. This could be due to the suggestion that $GABA_B$ receptors within insects, similarly to RDL $GABA_A$ receptors, have different properties to mammalian $GABA_B$ receptors (Lee et al., 2003). This would explain as to why, whereas in mice, baclofen is able to interact with mammalian $GABA_B$ receptors (Chu et al., 1990), the same is not observed in invertebrates.

To observe whether baclofen changed the previously seen ethanol-induced decrease in *L. variegatus* movement, *L. variegatus* were pre-treated with baclofen and their behavioural response to increasing concentrations of ethanol observed. When observing stereotypical movement, during both body reversal and helical swimming at 100 mM ethanol, *L. variegatus* displayed a significant change in behaviour when pre-treated with baclofen (Figure 3.12. A & B). This however was not observed at any other concentrations of ethanol and was not observed at all when observing free locomotory behaviour (Figure 3.13. B). Whilst this could again be explained by a GABA receptor pathway only being present in the glutamatergic-mediated giant interneuron pathways that are responsible for stereotypical movement and not free locomotion, within the human brain, baclofen has been demonstrated to inhibit glutamate release (Babcock et al., 2002) and reduce glutamate's excitatory strength (Yamada et al., 1999) which suggests that this reversal of ethanol-induced decrease in stereotypical movement is not due to alteration in glutamate activity by baclofen. In rat brains, ethanol has been shown to increase $GABA_B$ receptor protein expression which was significantly reversed by 10 mg/kg baclofen administration (Li et al., 2005). Although this provides limited insight into why there was no change in the free locomotory response to ethanol, it may be that the 20 mM administration of baclofen was enough to reverse the effects of 100 mM ethanol, but not enough to reverse the effects of 250 mM and 500 mM ethanol. Clinical studies using baclofen have reported that $GABA_B$ receptors in alcohol dependent individuals show reduced sensitivity to baclofen and require higher doses to feel any therapeutic effect (Durant et al., 2018). As our data demonstrated that the highest concentration of baclofen administered alone of 20 mM showed no significant change to *L. variegatus* behaviour, it could be suggested that observing the effects of higher concentrations of baclofen alone and with ethanol may provide us with more insight. As the off-license therapeutic use of baclofen aims to reduce the consumption of ethanol by reducing the reinforcing effects experienced during withdrawal

such as anxiety and cravings (Addolorato, 2002), it may explain as to the lack of change in ethanol-induced movement. The effect of baclofen on ethanol consumption in *L. variegatus* may be better observed using a place preference assay, through which the preference of chronically administered *L. variegatus* to ethanol could be compared with and without baclofen treatment. This could be completed using a CPP method that has been adapted for zebrafish (Mathur, Lau & Guo, 2011; Brock et al., 2017). As *L. variegatus* demonstrate a sensitivity to light (Daoud et al., 2022), worms cultured in ethanol would be conditioned to associate an ethanol environment with light and pondwater with the dark. By comparing whether ethanol cultured worms pre-treated with baclofen are more likely to swim towards the light or dark compared to ethanol cultured worms non-treated with baclofen, this could illustrate whether baclofen has any effect on chronic ethanol administration in *L. variegatus* and their preference to alcohol.

Whilst $GABA_A$ and $GABA_B$ receptor pathways have been widely researched in both vertebrates and invertebrates, there is another GABA receptor, known as $GABA_C$ (Bormann & Feigenspan, 2001). Whilst its structure as a ligand gated ion channel is the same as $GABA_A$, its pharmacological profile is different (Enz & Cutting, 1998). $GABA_C$ receptors show a higher sensitivity to GABA compared to $GABA_A$ receptors (Feigenspan & Bormann, 1994) and are also insensitive to bicuculline (Bormann & Feigenspan, 2001). It may be theorised that the differences observed between mammalian and invertebrate $GABA_A$ and $GABA_B$ receptors are also seen in $GABA_C$ receptors which could explain the actions of ethanol, GABA, bicuculline and baclofen in *L. variegatus*.

5.8. Nicotine

To further elucidate *L. variegatus* as an *in vivo* model for substances of abuse, we wanted to expose *L. variegatus* to nicotine, another psychoactive compound used commonly as a recreational drug of abuse.

Administering increasing nicotine concentrations resulted in body reversal and helical swimming both being dose-dependently reduced (Appendix Figure 6 A&B), which persisted even 10 minutes after nicotine removal (Appendix Figure 6 C & D), unlike the previous drugs

with which recovery in movement was seen 10 minutes following drug removal. The same dose-dependent decrease in behaviour was seen in free locomotory movement (Appendix Figure 6 F) where similar to stereotypical movement, this persisted 10 minutes after nicotine removal (Appendix Figure 6 G) and at 0.25 mM nicotine, 24 hours after nicotine removal (Appendix Figure 6 G). Our results are similar to previous work carried out in L*. variegatus* by Lesiuk & Drewes (1999b), who demonstrated that at concentrations of ≥0.5 mM, nicotine administration results in a significant decrease in pulse rate (beats/min). These results differ, however, to nicotine administration in other models. Nicotine administered to *C. elegans*, whilst demonstrating a brief hypoactivity, actually results in a dose-dependent increase in locomotory behaviour which starts at 4 minutes (Feng et al., 2006). Although we expose *L. variegatus* to nicotine for 10 minutes and still observe a decrease in movement, it would be beneficial to observe the time point at which nicotine starts to exert its effect, like we have already done with ethanol. The nicotine-induced increase in movement seen in *C. elegans* follows nicotine's pharmacological profile as a stimulant (Benowitz, 2009), also seen in vertebrate models such as mice (McCarthy et al., 2018).

As we explored for ethanol, we wanted to explore the *in vitro* response of *L. variegatus* to nicotine via the expression of Hsp70, Hsp60 and Cytochrome C. Unlike our observations for ethanol, *L. variegatus* Hsp 70, Hsp60 and Cytochrome C expression did not change following nicotine treatment (Figure 4.2.). Hsp70 expression has been shown to both increase and decrease following nicotine exposure, depending on where it is being induced. Nicotine administered to oral mucosal keratinocytes reduced the expression of Hsp70 and increased cell death with the authors suggesting that nicotine is involved in the pathogenesis of the mouth disease, oral lichen planus (Sheykhbahaei et al., 2021) however blood samples from smokers have shown significantly raised extracellular Hsp70 levels compared to non-smokers (Santos et al., 2018). This highlights a limitation in studying total protein in *L. variegatus* as it does not allow for the observation of any localised changes of protein expression in specific organs or tissues. Hsp60 levels are also upregulated when endothelial cells are exposed to cigarette smoke (Kreutmayer et al., 2011) however there is no data available to confirm whether this upregulation is seen due to nicotine or other components of cigarette smoke.

5.9. Cholinergic system

The cholinergic system is another receptor pathway identified as mediating the effects of drugs of abuse such as ethanol and nicotine (Calarco & Picciotto, 2020; Davis & de Fiebre, 2006). As with the selected proteins of the GABAergic system, we sought to determine the percentage similarities of the available protein sequences of all the muscarinic and nicotinic subunits in the genera *Homo sapiens*, *Rattus*, *Mus*, *Xenopus* and *Caenorhabditis*, to observe conservation of these subunits from *Homo sapiens* in other animal models, starting from the genus *Rattus* down to *Caenorhabditis*.

We observed that all muscarinic subunits, with the exception of M4, are conserved down to *C. elegans* (Table 4.1.). In *C. elegans*, the mAChR M1/M3/M5 homolog GAR-3 is found asymmetrically on the dorsal motor neuron cell bodies (Chan et al., 2013). When ACh is released, it interacts with GAR-3 and generates a signal that activates the presynaptic voltage-gated Ca^{2+} ion channels which then promotes the release of other neurotransmitters (Chan et al., 2013). GAR-3 exhibits the same function as the *Homo sapiens* M1, M3 and M5 mAChR subunits which are responsible for the excitatory actions of ACh compared to M2 and M4, which are responsible for the inhibitory actions of ACh (Felder, 1995). GAR-2 is the homolog for the mAChR M2 subunit in *C. elegans* and opposite to GAR-3, when ACh interacts with GAR-2, neurotransmitter release at neuromuscular junctions is inhibited (Lee et al., 2000).

Data to calculate all nicotinic subunit conservations was much more limited. Nicotinic β2 and β4 showed conservation down from *Rattus* to *Caenorhabditis*, with the vertebrates *Rattus* and *Mus* showing >90% sequence conservation compared to *Homo sapiens*. Nicotinic α4 also showed conservation however this was only down to *Xenopus* but like β2 and β4, *Rattus* and *Mus* showed >90% sequence conservation. The nicotinic α4 and β2 subunits being highly conserved in rodent models is supported by electrophysiological studies showing that within the human brain, nAChRs containing the α4β2 subunits show the highest affinity to nicotine (Dani, 2015). Nicotinic α7 showed conservation in *Caenorhabditis* and despite full protein sequences for *Rattus* to *Xenopus* being unavailable, studies have demonstrated that *Rattus*, *Mus* and *Xenopus* all express nicotinic α7 subunits (Cao et al., 2021; Palma et al., 1996; Tribollet et al., 2004). Genes encoding the nAChR α7 subunit are also present in *Drosophila*

and show high homology to the vertebrate α7 subunits (Velazquez-Ulloa, 2017). Despite protein sequences for many of the nAChRs not being available for *Caenorhabditis,* the nAChR gene families in *C. elegans* consists of 27 subunits, making it one of the most extensive and diverse nAChR gene families (Jones & Sattelle, 2004).

Acetylcholine is a major excitatory neurotransmitter which exerts its effects through mAChRs and nAChRs in both vertebrate (Colangelo et al., 2019) and invertebrate (Richmond & Jorgensen, 1999) models. To identify the presence of an cholinergic system within *L. variegatus*, a commercial kit was used to calculate the endogenous acetylcholine levels and activity of endogenous acetylcholine esterase within the worms. We were able to confirm the presence of endogenous acetylcholine (Figure 4.1. A) and acetylcholine esterase (Figure 4.1. B), indicative of a cholinergic system within this. Not only does identifying components of an acetylcholine system within *L. variegatus* help us understand its physiology but as *C. elegans* also have an endogenous acetylcholine system (Pereira et al., 2015), it allows comparison of both models and expands our understanding of invertebrate models and their role in pharmaceutical research.

5.10. Nicotinic acetylcholine receptor pathway

To further elucidate the cholinergic system present within *L. variegatus*, we observed the behavioural responses to nAChR antagonists mecamylamine and tubocurarine. Administration of increasing concentrations of mecamylamine had no significant change on the body reversal, helical swimming (Figure 4.3. A & B) and free locomotory movement (Figure 4.3. F) of *L. variegatus* during the 10-minute drug exposure period, however, 10 minutes following the removal of 50 μM mecamylamine, there was a significant decrease in both stereotypical movements (Figure 4.3. C & D), which was not observed at 100 μM. Whilst there is no direct evidence which explains why this reduction in behaviour was not seen at 100 μM, there is evidence which suggests that mecamylamine can take from 10-30 mins to leave the nAChR, demonstrated by Donnelly (2009), who showed that ACh currents and therefore action potentials were blocked 10-mins following 50 μM mecamylamine removal, which would explain a reduced stereotypical movement response in *L. variegatus* 10-mins following 50 μM mecamylamine removal. Additionally, in human brains, low concentrations of

mecamylamine, <20 μM, inhibit the nAChRs that are located on the glutamatergic dorsal raphe nucleus terminals (Hernández-González et al., 2020). Glutamatergic signals are responsible for *L. variegatus* stereotypical movement and therefore, it could be hypothesised that the reduction in *L. variegatus* stereotypical movement was due to mecamylamine acting on nAChRs located in the glutamatergic-mediated giant interneuron pathways (Lybrand et al., 2020). Whilst it does not explain why there was no change in movement seen during drug exposure, all of this data may explain why 10-mins following 50 μM mecamylamine removal, there was a significant decrease in both body reversal and helical swimming. Data regarding the behavioural responses of invertebrate models to mecamylamine administration is limited however in mice, the administration of mecamylamine dose-dependently reduced licking behaviour in a conditioning task and locomotory behaviour in an open-field task (Kaneko et al., 2022).

Administration of increasing concentrations of tubocurarine dose-dependently decreased both *L. variegatus* helical swimming and body reversal (Figure 4.4. A & B) however did not have any significant impact on *L. variegatus* free locomotory behaviour (Figure 4.4. F). Tubocurarine is a muscle relaxant which blocks neuromuscular transmission (Bowman, 2006) as shown in *C. elegans*, where tubocurarine administration results in a decrease in the frequencies of Ca^{2+} currents and therefore action potentials (APs) at gap junctions (Liu, Chen, et al., 2011). This could explain the decrease in *L. variegatus* stereotypical movement as the lack of release of action potentials mean that the worms are unable to recoil away from any tactile stimulation. The lack of tubocurarine's effect in free locomotory behaviour could be explained by AP firing continuing despite the lack of neural input which is seen in *C. elegans* (Liu et al., 2011). Following the removal of nicotinic receptors in *C. elegans*, there was still some locomotory movement observed and even though the frequencies of Ca^{2+} currents and APs were reduced, tubocurarine had no effect on their synchronicity meaning that the mechanism required for body muscle movement remained intact (Liu, Chen, et al., 2011). It was also shown in one study that tubocurarine administered to unstimulated squid nerve fibres both induced and blocked short periods of hyperpolarisation which, whilst the induction would lead to reduced action potential firing and reduced movement, the blockage would explain normal action potential firing and therefore normal movement (Villegas, 1973).

Mecamylamine has been researched as a treatment option for smoking cessation, with multiple trials showing that smokers are more likely to abstain when administered mecamylamine due to fewer cravings and less withdrawal symptoms (Lancaster & Stead, 1998; Rose et al., 1994b, 1998). To help us understand the influence of the competitive nAChR antagonists on nicotine-induced *L. variegatus* movement, mecamylamine and tubocurarine were administered in two ways with nicotine: via pre-treatment before nicotine exposure or co-administration with nicotine exposure.

L. variegatus that were pre-treated with mecamylamine and then exposed to nicotine showed an increase in free locomotory movement compared to *L. variegatus* that were exposed to nicotine alone (Figure 4.5.B). In *C. elegans*, mecamylamine administration blocks the actions of nicotine however as nicotine has a stimulatory effect in *C. elegans*, mecamylamine induces lower locomotory behaviour compared to the administration of nicotine alone (Sellings et al., 2013). Pre-treatment with mecamylamine has also been shown to reduce nicotine-seeking behaviour where mecamylamine pre-treated rats responded to the nicotine-associated visual cue less than rats who were not pre-treated (Liu et al., 2006) and also reduced nicotine self-administration (Liu et al., 2007). Tubocurarine pre-treatment did not change the effects of nicotine during exposure and the inhibitory effects of nicotine were still observed 10 minutes following nicotine removal (Figure 4.5.B). Mecamylamine and tubocurarine were administered at differing concentrations due to their NOAELs and therefore we hypothesise that this may explain the differences in the behavioural response as there may have been too much competition between tubocurarine and nicotine to allow tubocurarine to have any impact.

Co-administration of mecamylamine and tubocurarine with nicotine did not result in any changes in free locomotory behaviour compared to the administration of nicotine alone (Figure 4.6.). Zambrano et al., (2015) demonstrated that a twice daily co-administration of nicotine and mecamylamine results in an additive upregulation of α4β2 nAChRs within embryonic mouse brain cells compared to nicotine alone and that chronic mecamylamine treatment increased nicotinic binding to nAChRs.

Differences between the actions of co-administration compared to pre-treatment with mecamylamine and its effects on nicotine have been demonstrated in rats with opposite effects to what we have observed in *L. variegatus*. Nicotine induces an increase in dopamine and its metabolites in the nucleus accumbens but when mecamylamine is co-administered with nicotine, this nicotine-induced increase in dopamine is reduced (Nisell et al., 1994). When mecamylamine is administered as a pre-treatment, however, there is no effect on the nicotine-induced dopamine increase (Nisell et al., 1994).

As a novel *in vivo* model, *Lumbriculus variegatus* has demonstrated both its strengths and weaknesses for use in pharmacological and toxicological studies. Whilst a limitation of using these worms is the current inability to observe drug effects on isolated organs and tissues, a whole organism response is still able to be observed both *in vivo* and *in vitro* as shown during this study. Behavioural responses of *L. variegatus* are able to be easily observed quantified and experimental methods such as extraction of proteins and fatty acids have proven successful in *L. variegatus.* Whilst in this study, there may not have been drug responses relevant to what has been observed in vertebrate models and humans, the success of completing these techniques in *L. variegatus* further strengthens the worms in its use as a tool to establish drug response relationships for a wider variety of drugs.

6. Future Directions

Throughout this project, we have aimed to explore two highly used drugs of abuse, ethanol and nicotine, and the receptor pathways through which they exert their mechanism of action.

One characteristic of drug addiction is "drug-seeking behaviour" where an individual will compulsively go out of their way, despite any harm that it may bring to their personal and social wellbeing, to find drugs to consume (Everitt, 2014). Replicating this behaviour in animal models is possible using a technique known as conditioned place preference (CPP) which has been used for both nicotine and ethanol (Bechtholt & Cunningham, 2005; Natarajan et al., 2011). Invertebrate models such as *C. elegans, D. melanogaster* and *P. clarkii* have demonstrated CPP for ethanol and nicotine (Engleman et al., 2018; Gutierrez et al., 2022; Kaun et al., 2012; Lee et al., 2009) highlighting that invertebrate models can demonstrate complex behaviour. One of the next steps would be to utilise a place preference assay for the use of *L. variegatus*. Not only would this to allow us to observe whether the worms show preference for ethanol and nicotine, it would also allow us to explore the receptor pathways that ethanol and nicotine have been suggested to mediate their effects through. For example, in mice, bicuculline administration was used within a CPP model to demonstrate the role of the $GABA_A$ receptor pathway in the reinforcing effects of ethanol (Chester & Cunningham, 1999). We could use this same principle in *L. variegatus* with the other receptor agonists and antagonists that we have previously explored (bicuculline, baclofen, mecamylamine and tubocurarine) to further elucidate the reinforcing properties of ethanol and nicotine.

Nicotine has been shown to demonstrate both acute and chronic tolerance in humans (Perkins, 2002; Zuo et al., 2011) and its inhibitory effects on movement in *L. variegatus* persist even 10 minutes after drug removal therefore, like we have explored for ethanol, our next step in studying drug tolerance would be to observe whether *L. variegatus* develop acute or chronic nicotine tolerance using the same methodology developed for ethanol. To explore acute tolerance, *L. variegatus* would be exposed to 1 mM nicotine and to explore chronic tolerance, L. variegatus would be cultured in 0.01 mM nicotine. *C. elegans* have been demonstrated to develop a time-dependent tolerance to nicotine following chronic exposure

(Feng et al., 2006; Polli et al., 2015) with Polli et al., (2015) further demonstrating that the up-regulation of nAChRs seen in humans during the development of chronic nicotine tolerance (Wonnacott, 1990) is also observed in *C. elegans*. Furthermore, a daily co-administration of nAChR antagonist mecamylamine with nicotine for 6 days blocked the development of tolerance in rats (McCallum et al., 2000) and therefore this would be something we could replicate with *L. variegatus* using the nicotine culture, especially since our results from this study have demonstrated that co-administration of mecamylamine with nicotine does not impact *L. variegatus* behaviour following acute nicotine exposure.

To complete the work in elucidating the behavioural responses of *L. variegatus* to the $GABA_B$ receptor agonist baclofen, we would carry out the comparison stereotypical movement and free locomotory assays with GABA that have been previously completed with bicuculline. During these assays, we would pre-treat the worms with baclofen and compare the behavioural responses of GABA with baclofen pre-treatment compared to baclofen alone. This would provide a further insight into the presence of an *L. variegatus* $GABA_B$ receptor pathway. Whilst behavioural studies comparing the behavioural responses to GABA with and without pre-treatment of baclofen are not available, using rat brain slices, baclofen has been shown to inhibit excitatory signals from the glutamatergic system and inhibitory signals from the GABAergic system via activation of pre-synaptic $GABA_B$ receptors (Yamada et al., 1999). We would hypothesise that baclofen pre-treatment would inhibit the GABA-induced decrease in observed in *L. variegatus* stereotypical movement.

In the UK, it is estimated that 58% of patients presenting with a risk of alcohol dependence also smoke, with the level of smoking increasing as alcohol consumption increases (Garnett et al., 2022). Co-administration of nicotine with ethanol potentiates the rewarding effects of the mesolimbic reward pathway as a significantly greater amount of dopamine is released within the nucleus accumbens (NAc) compared to nicotine or ethanol alone (Waiess et al., 2019). Vertebrate models, including humans and rodents, also demonstrate a behavioural response when nicotine and ethanol are co-administered as alcohol self-administration increases in both non-dependent and dependent individuals (Leao et al., 2015; Olausson et al., 2001). There is no existing data within invertebrate models comparing the behavioural response of co-administrating nicotine and ethanol compared to administrating these drugs alone and so

we aim to administer both drugs together using both the stereotypical movement and free locomotory assays conducted in the same way as the nicotine co-administration assay to illustrate whether there is any novel response. Once a place preference assay is able to be established, it would also be insightful to observe whether pre-treatment or co-administration of nicotine with ethanol demonstrates any change in ethanol-seeking behaviour.

Using a commercial kit, we would also aim to establish the internal blood alcohol concentration (BAC) of *L. variegatus* to determine how much ethanol the worms ingest and whether ethanol enters via the mouth or via diffusion. When calculating the internal concentration of ethanol in *C. elegans*, Lee et al., (2009) found that that the external ethanol concentration was higher than the internal ethanol concentration. Similar results were also demonstrated by Mitchell et al., (2008) who proposed that ingestion of ethanol likely occurs through the body however Lee et al., (2009) suggest that the cuticle has low permeability to ethanol, explaining the lower internal ethanol concentration. It was also determined that to induce *C. elegans* ethanol preference, the internal concentration would have to be over 300 mM (Lee et al., 2009). It would be interesting to observe at what internal concentration, if any, that *L. variegatus* would demonstrate ethanol preference.

L. variegatus' ability to undergo regeneration has meant that it's one of the earliest annelids to be used to model regeneration (Acosta et al., 2021), with Bonnet (1745) demonstrating that one worm cut up into 16 pieces can regenerate from each one of those pieces. This regeneration process occurs in five stages: 1) wound healing, 2) blastema formation, 3) blastema patterning, 4) resegmentation and 5) growth. This regenerative ability is used by the worms to undergo autotomy or asexual fragmentation (Zattara & Bely, 2016). Exposure to toxic compounds has been shown to impact *L. variegatus* regeneration with copper increasing the likelihood of *L. variegatus* engaging in fragmentation (O'Gara et al., 2004) and microplastics bisphenol A (BPA) and bisphenol S inhibiting the first stages of regeneration (Wang & Wang, 2021; Vought & Wang, 2018). However, there is no available data observing the effects of drugs such as ethanol and nicotine on *L. variegatus* regeneration. Ethanol exposure has been demonstrated to delay head regeneration of the flatworm *Schmidtea mediterranea* (Lowe et al., 2015). It was suggested these results could allow insight into the effects of ethanol on conserved neurodevelopmental processes that lead to Foetal Alcohol

Spectrum Disorder in humans (Lowe et al., 2015). There is no data available for the effect nicotine exposure may have on regeneration and therefore, an insightful next step would be to determine how long the regeneration process takes for *L. variegatus* and then optimising existing regeneration assays, such as that used by Martinez et al., (2008), to observe how regeneration is impacted following both acute and chronic exposure of *L. variegatus* to ethanol and nicotine.

Studies have also demonstrated that alongside affecting regeneration, toxic compounds can also affect the DBV pulse rate of *L. variegatus*. Wang & Wang (2021) used BPA to observe its impact on *L. variegatus* pulse rate, where they demonstrated that both acute and long-term exposure to BPA increased the DBV pulse rate. Older studies have demonstrated that acute nicotine exposure decreases the *L. variegatus* pulse rate (Lesiuk & Drewes, 1999). These studies demonstrate techniques that can be used to quantify the pulse rate of *L. variegatus*, therefore allowing for it to be used as an endpoint in toxicity experiments. We would aim to optimise the pulse rate assays and use the drug exposure techniques that we have already optimised in the laboratory to observe the effects of both acute and chronic exposure of ethanol and nicotine on the pulse rate of *L. variegatus*, expanding its use as an invertebrate model for drugs of abuse. These are important to observe as both alcohol and nicotine consumption can result in cardiovascular complications (Benowitz & Burbank, 2016; Ginter & Simko, 2008) as demonstrated in vertebrate models, including humans, where both acute and chronic ethanol consumption can result in a lower heart rate (Fernández-Solà, 2020; Jones, 2005; Ryan & Howes 2002) and acute and chronic nicotine consumption can result in an increased heart rate (Benowitz & Burbank, 2016; Gajewska et al., 2014).

Ethanol and nicotine are two widely used substances of abuse across the world however there are also many more such as cannabinoids, opioids, and stimulants such as ecstasy and cocaine (McLellan, 2017). Within the UK, from 2020 to 2021, 89% of young people were in substance misuse treatment for cannabis and 21% were in treatment for misuse of ecstasy and cocaine (Office for Health Improvement and Disparities, 2022). To expand the use of *L. variegatus* an *in vivo* model for substances of abuse, we would aim to expose the worms to a wider variety of substances using the optimised stereotypical movement and free locomotory assays to observe their behavioural responses, building on the work available for the exposure of the

same drugs to *C. elegans*. *C. elegans* lack a homologous cannabinoid receptor pathway (van Es-Remers et al., 2022) and whilst data regarding Δ^9-tetrahydrocannabinol (THC) exposure is limited, cannabidiol (CBD) administered at physiologically relevant concentrations to *C. elegans* displayed no short-term or long-term toxicity (Land et al., 2021). Like we aimed to establish for GABA, using the optimised behavioural assays and exploring pre-treatment of either cannabinoid agonists such as delta 9-tetrahydrocannabinol or cannabinoid antagonists, such as Rimonabant, investigating the presence of a cannabinoid receptor pathway in *L. variegatus* would further establish this worm as an *in vivo* invertebrate model. The serotonergic system within *C. elegans* is more established and therefore the effect of drugs acting through this receptor pathway, such as cocaine (Ward et al., 2009) and ecstasy (Schreiber & McIntire, 2011) have also been well established, providing a foundation on which to build our work with *L. variegatus*.

The influence of genetics on both the acute response to drugs and the development of addiction following chronic consumption of drugs has been widely reported (Agrawal et al., 2012). With the genetic influence on the response to ethanol and nicotine being widely characterised in both *C. elegans* (Kwon et al., 2004; Feng et al., 2006; Smith. Jr et al., 2013; Johnson et al., 2017) and *Drosophila* (Wen et al., 2005; Velaquez et al., 2013; Larnerd et al., 2023), sequencing the genome of *L. variegatus* would widen its potential as a genomic model for substance abuse research. Although the genome of *Lumbriculus variegatus* has not been fully sequenced, extensive work has been done to make progress in this field, with the estimated genome size of *L. variegatus* being 2.64 Gbp (Tweeten & Morris, 2016), larger than the domestic mouse. Furthermore, the first regeneration transcriptome produced by Tellez-Garcia et al., (2021) when observing the genomic profile of regenerating *L. variegatus*, identified 136 transcripts that were likely to be expressed during worm regeneration. They also found that 73 of these could also code for proteins such as Hsp60. These results highlight not only the ability to sequence the genome of *L. variegatus*, but also identify parts of the genome that are comparable to other invertebrate, vertebrate and human models to further elucidate the genetic factors involved in drug response.

L. variegatus has shown its value as a model within educational settings. During the Multi-Institution Double Blind *In vivo* Trials (MIDBIT), the worms have been used by educational

institutions and their students to explore the behavioural responses of *L. variegatus* to a variety of drugs. They were first suggested for use in practical student sessions by Lesiuk & Drewes, (1999) to explore the effects of ethanol on pulse rate and SWIRL has since optimised the stereotypical movement assay for students to use in their practical classes. *L. variegatus* provide a major advantage over using vertebrate models as, whilst still ensuring ethical handling, not only do institutions not require any licensing for their use, they also do not require any specialist accommodations in which to house the worms. Students are able to observe the behavioural response in a whole model organism. It is necessary to keep optimising these techniques with *L. variegatus* and ensuring that students are able to engage with whole model organisms during their time in education.

7. Conclusion

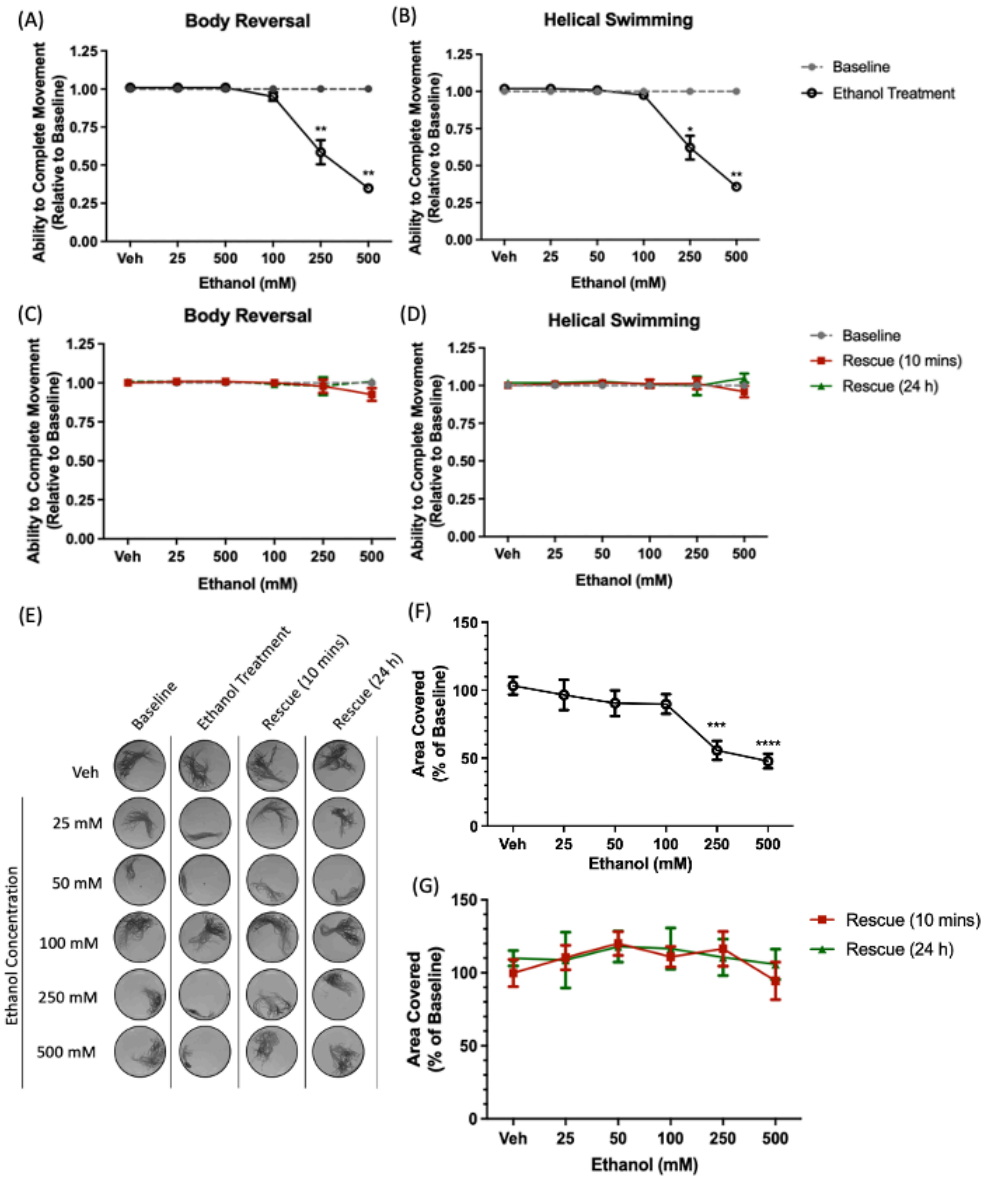
During this project, the use of *Lumbriculus variegatus* as an *in vivo* model in pharmacology was further established for its use in testing substances of abuse.

To build on the pharmacological profile of ethanol administration to *L. variegatus*, it has been established that the previously shown dose dependent ethanol-induced decrease in free locomotory movement begins at 2 minutes during exposure. *L. variegatus* also display a heat shock response which is initiated following 10-minute ethanol administration as shown by the increase in Hsp70 expression. Whilst *L. variegatus* develop acute functional tolerance to ethanol, they do not develop chronic ethanol tolerance. Chronic exposure to ethanol, however, does cause an increase in the body size of the worms. Whilst the presence of a GABA pathway in *L. variegatus* was suggested through a previously seen GABA-induced decrease in movement, it was unable to be established that the ethanol-induced decrease in *L. variegatus* movement was caused through this pathway.

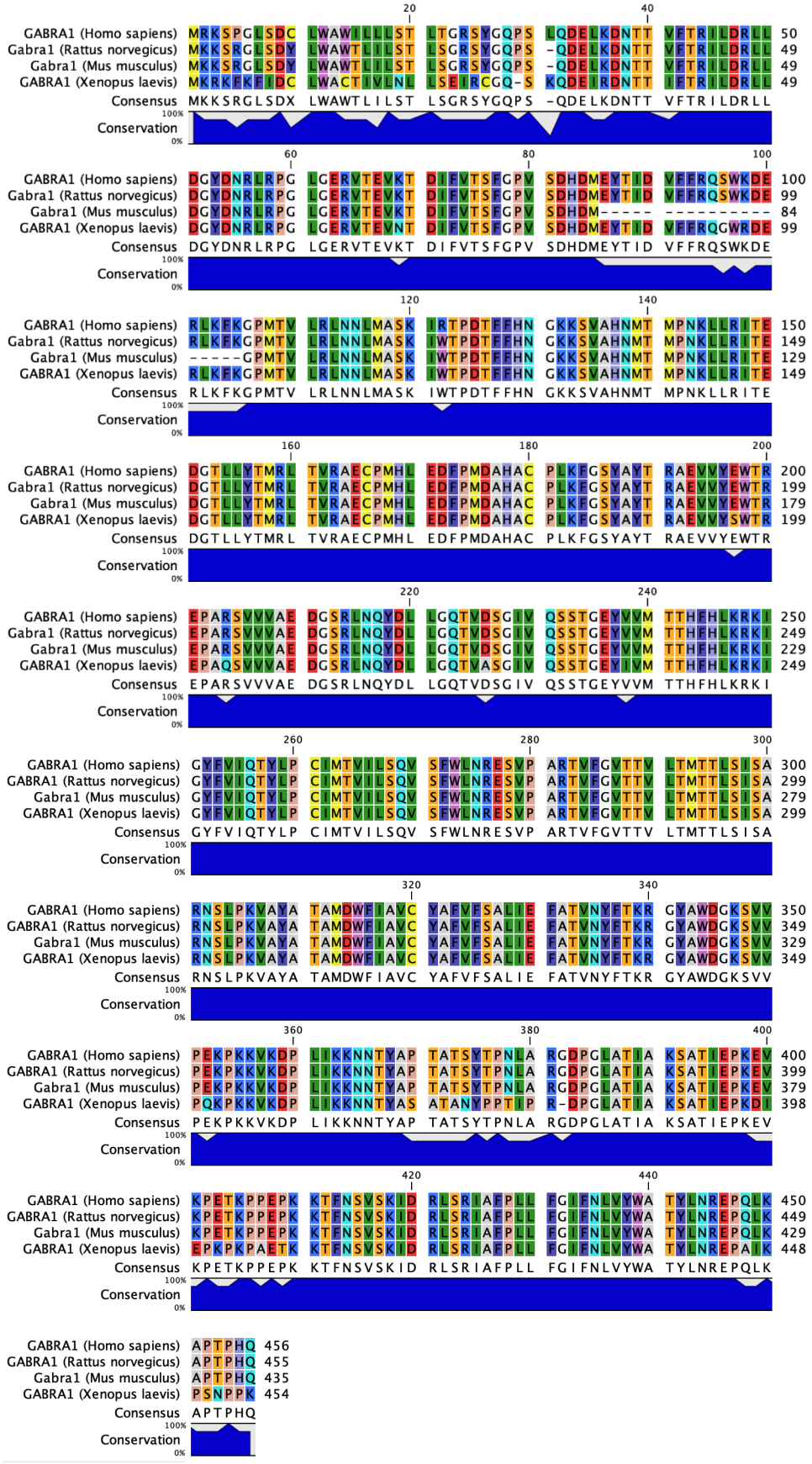
A pharmacological profile of nicotine administration to *L. variegatus* has also been developed. The activity of a cholinergic pathway was seen in *L. variegatus* by quantifying the presence of endogenous acetylcholine and acetylcholinesterase. Unlike ethanol, nicotine did not induce the heat shock response and the administration of nAChR antagonists mecamylamine and tubocurarine were able to alter the effect of the previously seen nicotine-induced decrease in *L. variegatus* movement, demonstrating the ability of nicotine to act through the cholinergic system in *L. variegatus*.

In future studies, it would be beneficial to further observe the impact of ethanol on a wider range of behavioural responses as well as quantify further *in vitro* observations, such as the blood alcohol concentration of *L. variegatus*. Establishing the timepoint at which nicotine is able to exert its effects and whether *L. variegatus* are able to develop tolerance to nicotine would further build the pharmacological profile that has been started in this study.

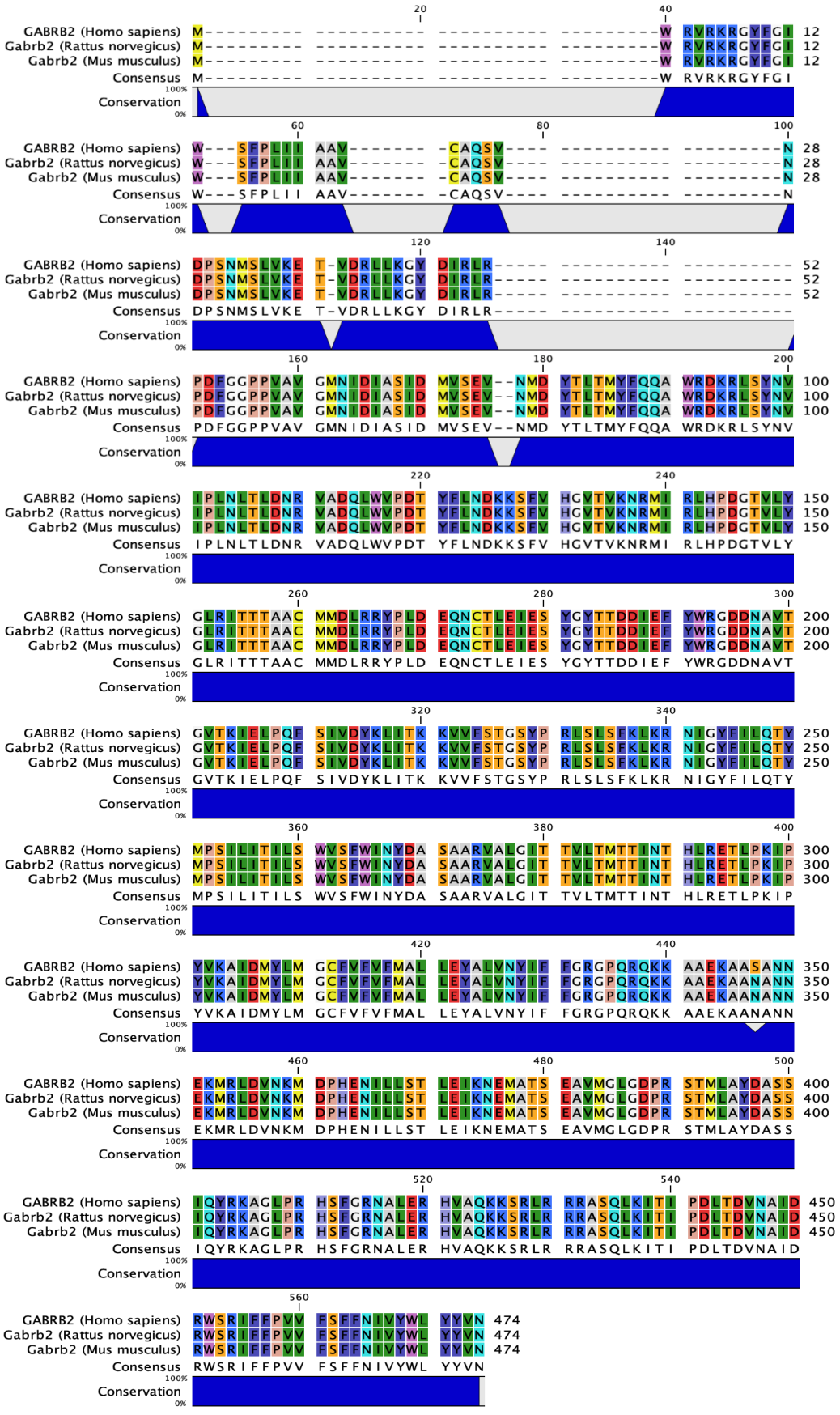
8. Appendix



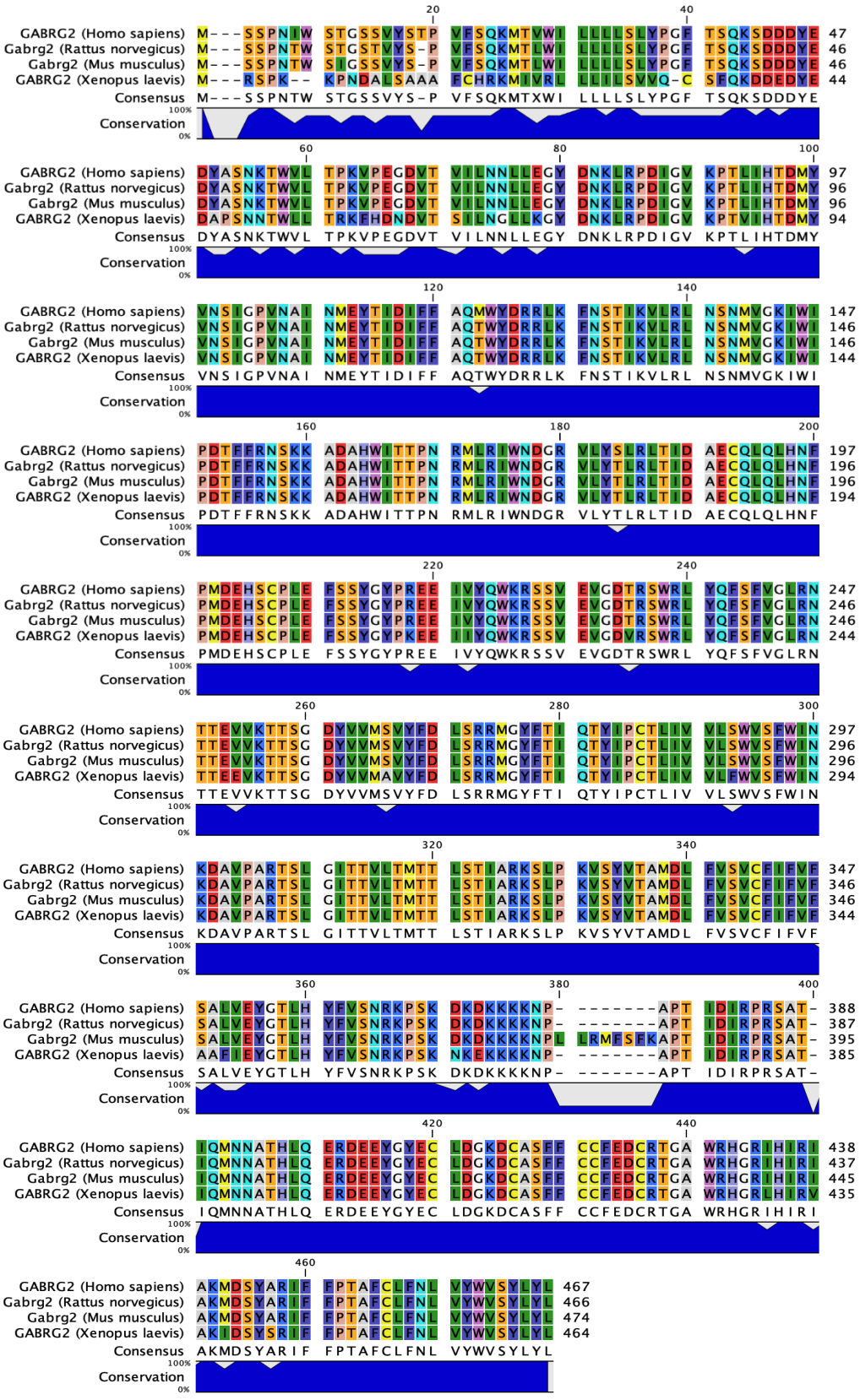
Appendix Figure 1. The effect of ethanol on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of ethanol (0 – 500 mM) and tested for the ability of tactile stimulation to elicit (A) body reversal or (B) helical swimming. Ethanol was then removed and the ability 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 0 – 500 mM ethanol, 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. Data is presented as a percentage of the area of baseline movement. Error bars represent the standard error of the mean n=8 for each concentration. Veh: artificial pondwater. *p=0.0156, **p=0.0078, ***p=0.0004, ****p<0.0001. Experimental repeats were conducted by Julanta Carriere and Shaurya Nathan Mathur. Taken from Bellamy (2020).



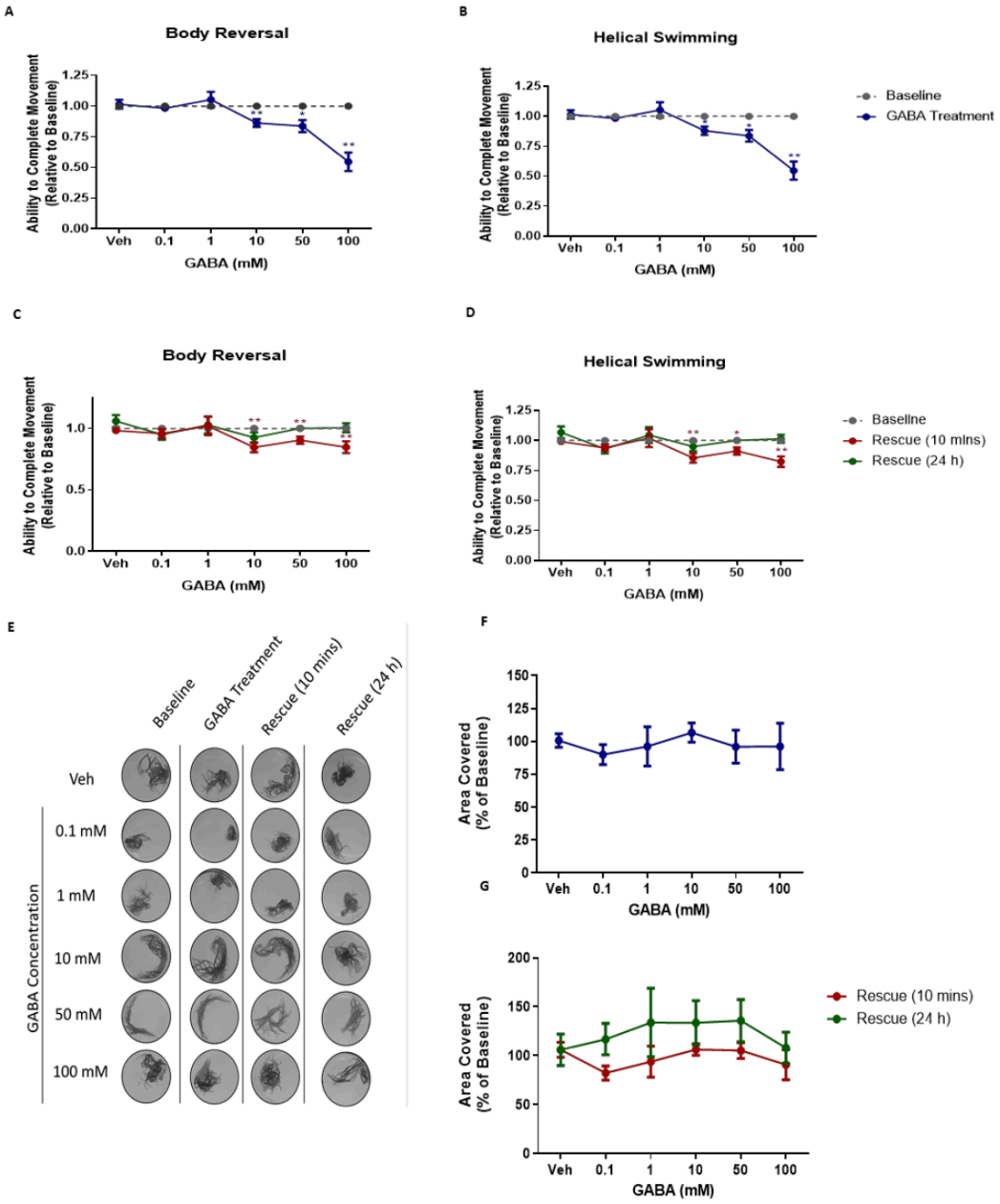
Appendix Figure 2. GABA$_A$ receptor subunit alpha 1 alignment.



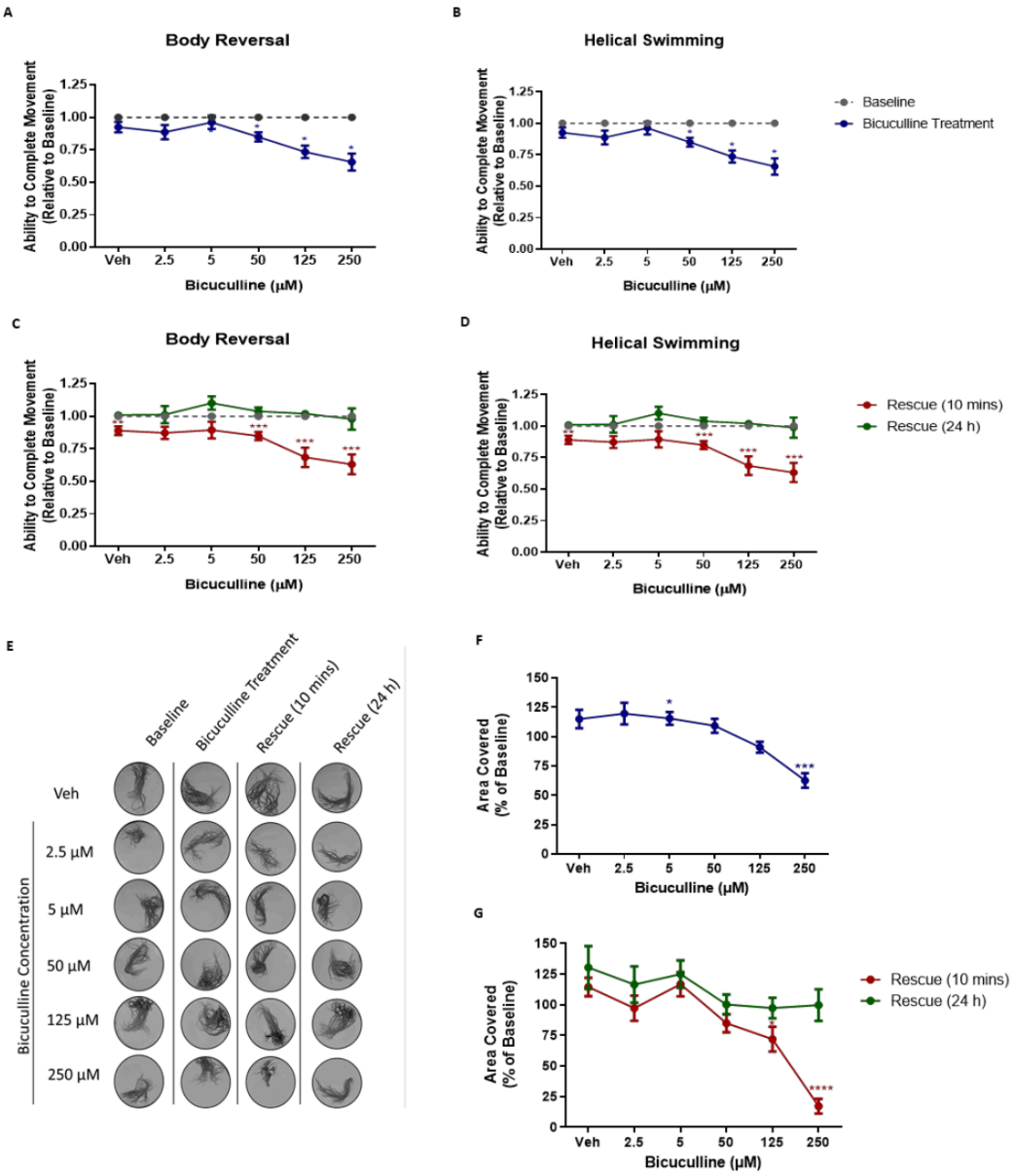
Appendix Figure 3. $GABA_A$ receptor subunit beta 2 alignment.



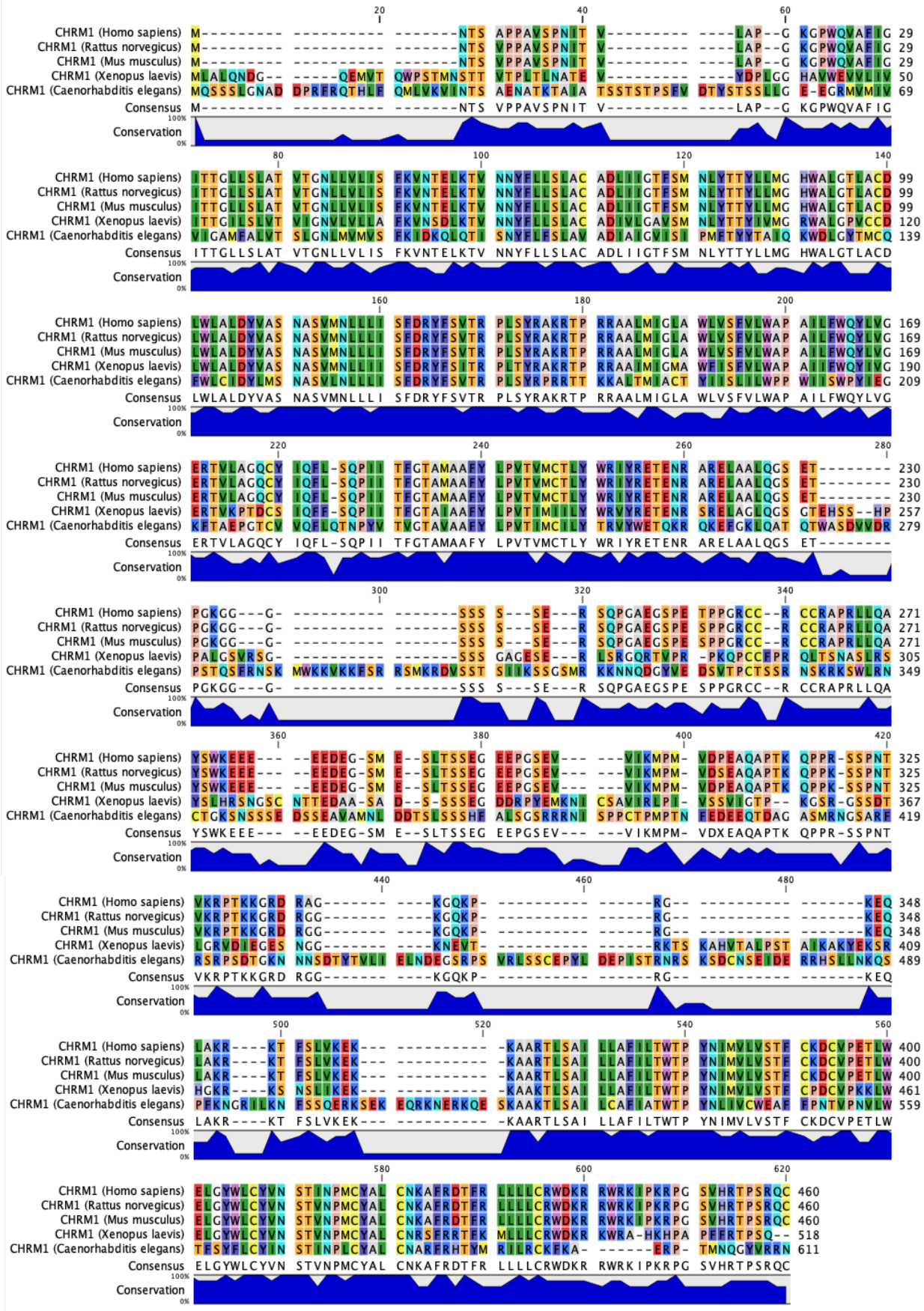
Appendix Figure 4. GABA$_A$ receptor subunit gamma 2 alignment.



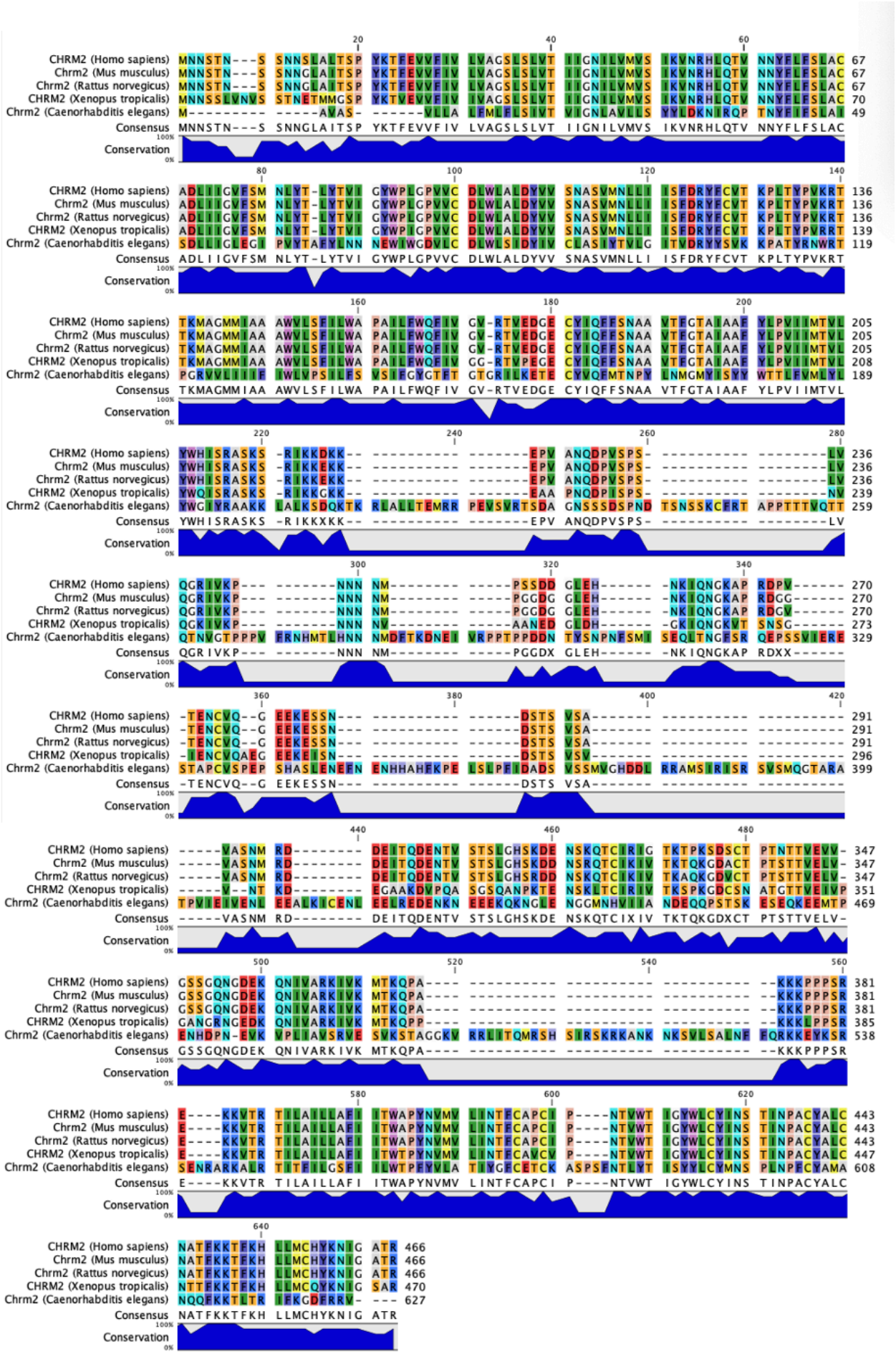
Appendix Figure 5. Effect of GABA on *Lumbriculus variegatus* behaviour. Increasing concentrations of 0.1 – 100 mM GABA were administered to *L. variegatus* and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. GABA was then removed and the ability 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. Data is presented as a percentage of the area of baseline movement. Error bars represent the standard error of the mean n=8 for each concentration. Veh: artificial pondwater. *p<0.05, **p<0.01. Experimental repeats were conducted by Caitlin Bellamy and Shaurya Nathan Mathur.



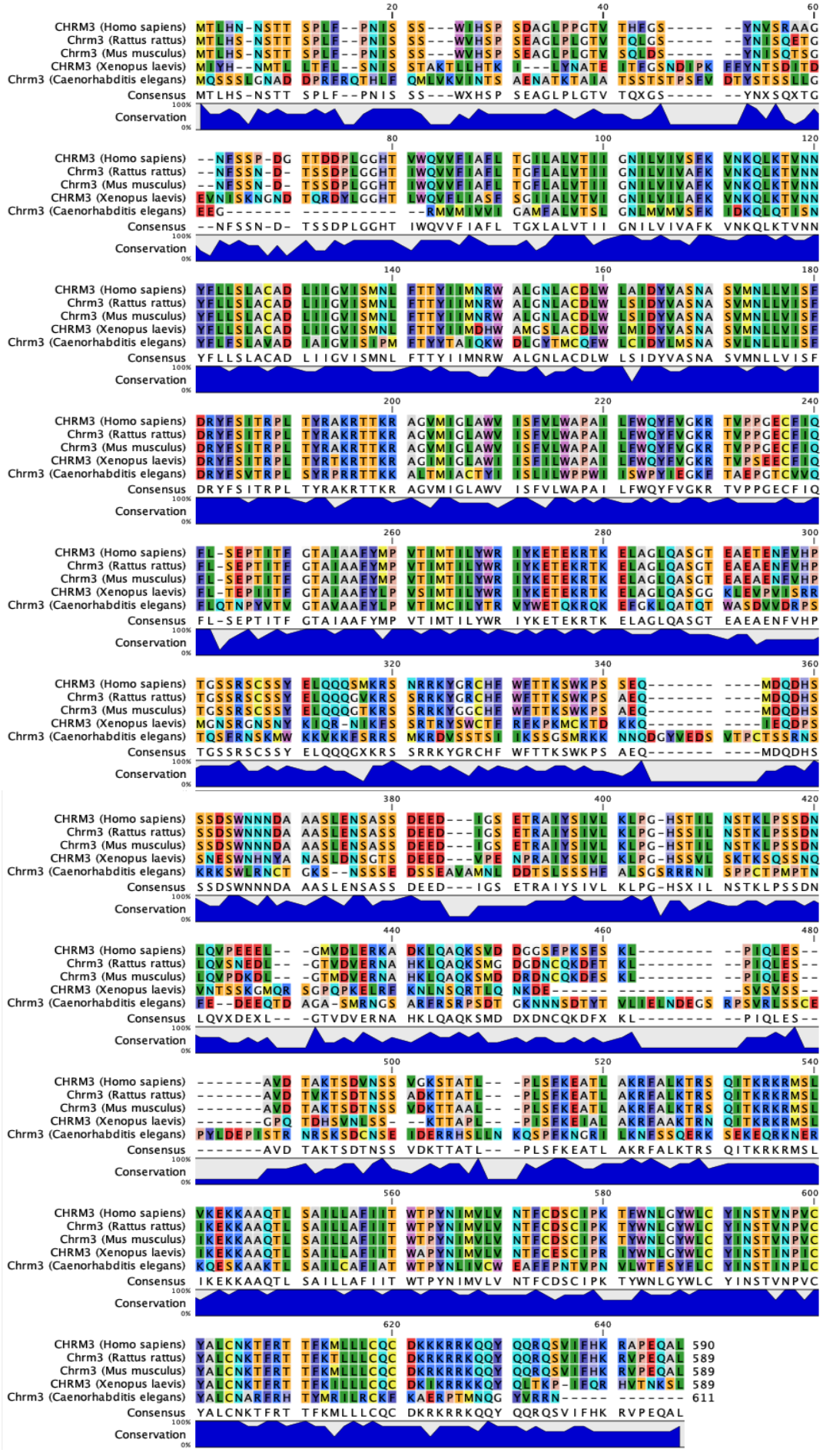
Appendix Figure 6. Effect of bicuculline on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of bicuculline (2.5 - 250 µM) and tested for the ability of tactile stimulation to elicit (A) body reversal or (B) helical swimming. Bicuculline was then removed and 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. Taken from Bellamy (2022).



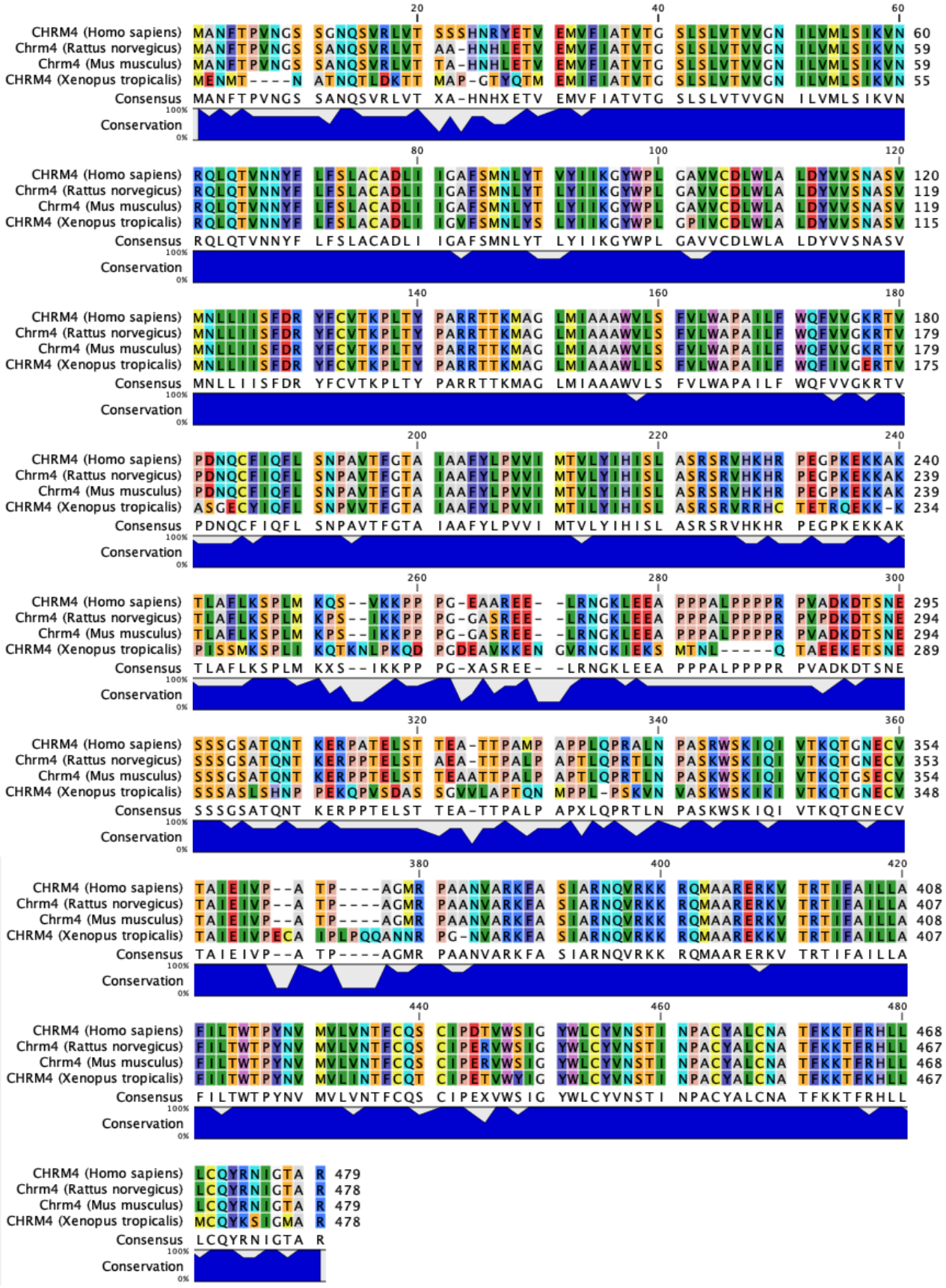
Appendix Figure 7. Muscarinic acetylcholine receptor 1 alignment.



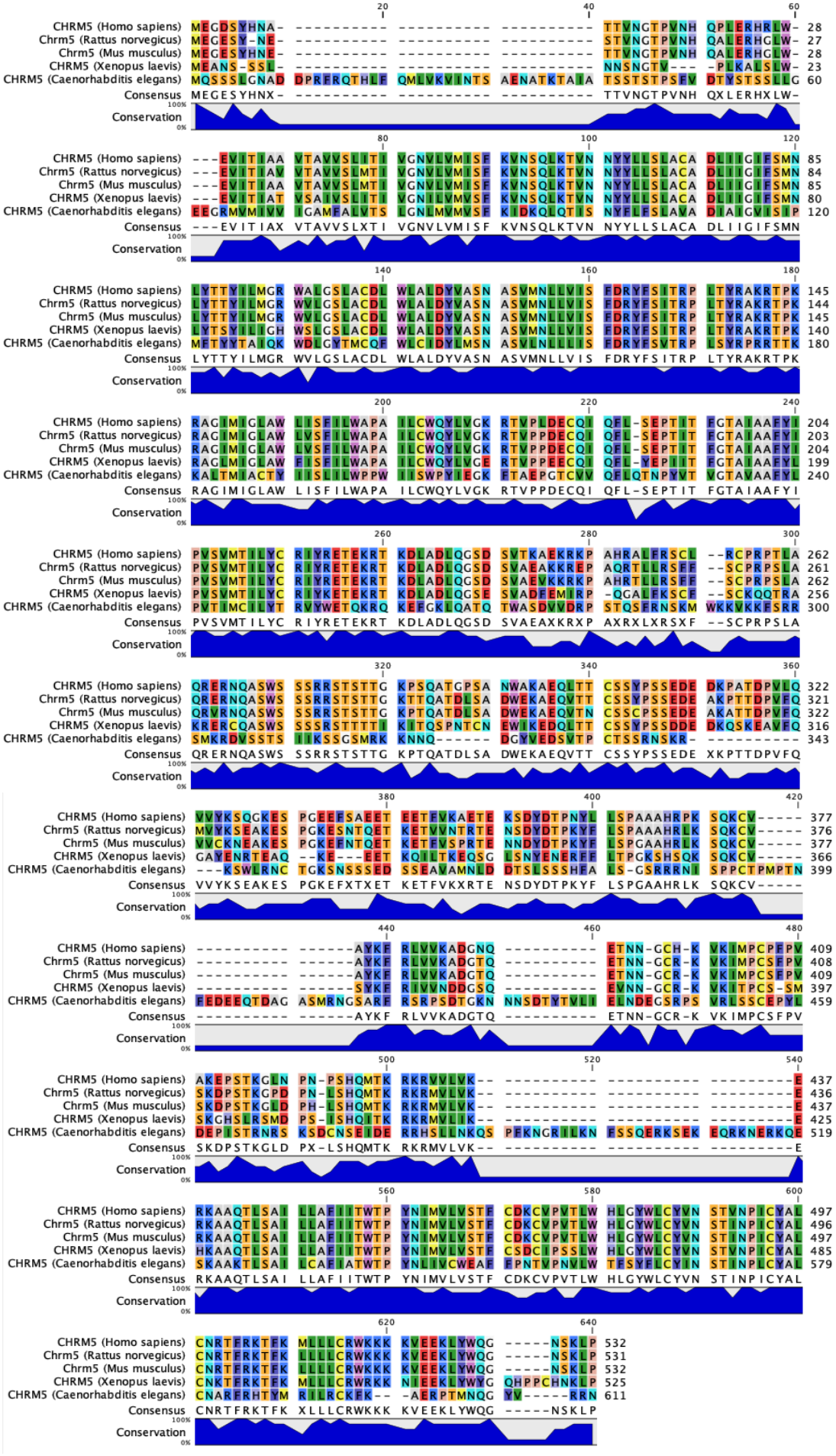
Appendix Figure 8. Muscarinic acetylcholine receptor 2 alignment.



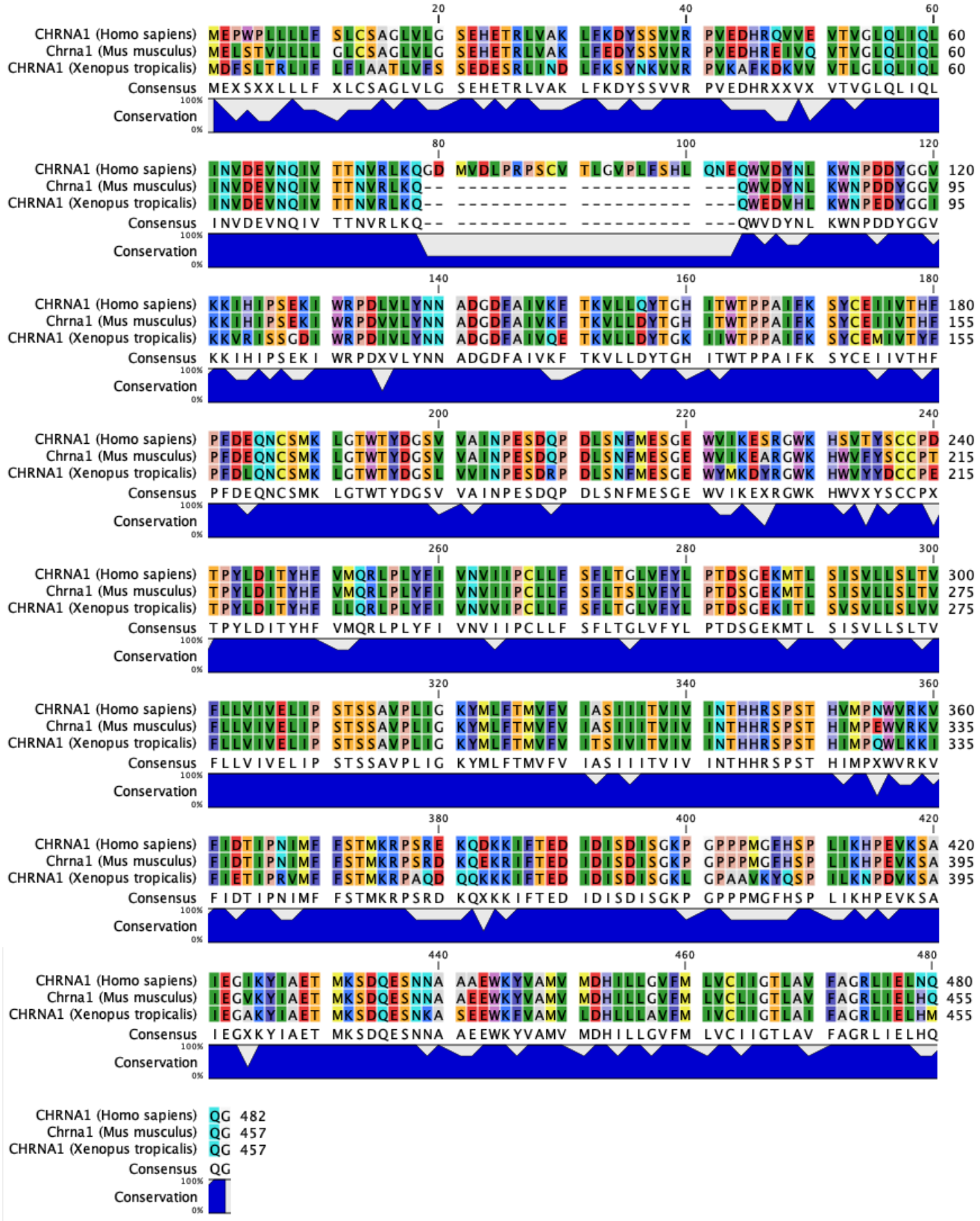
Appendix Figure 9. Muscarinic acetylcholine receptor 3 alignment.



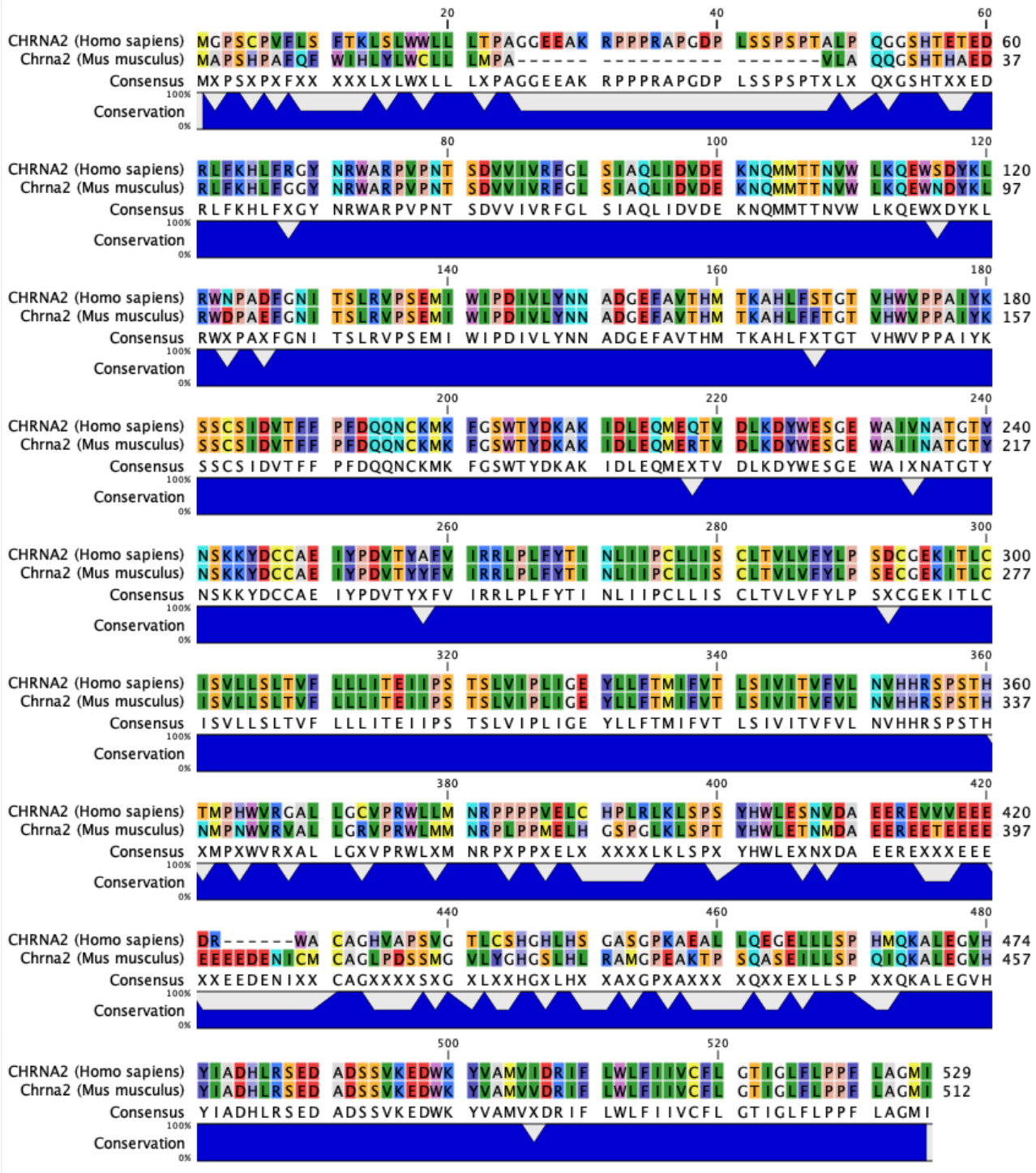
Appendix Figure 10. Muscarinic acetylcholine receptor 4 alignment.



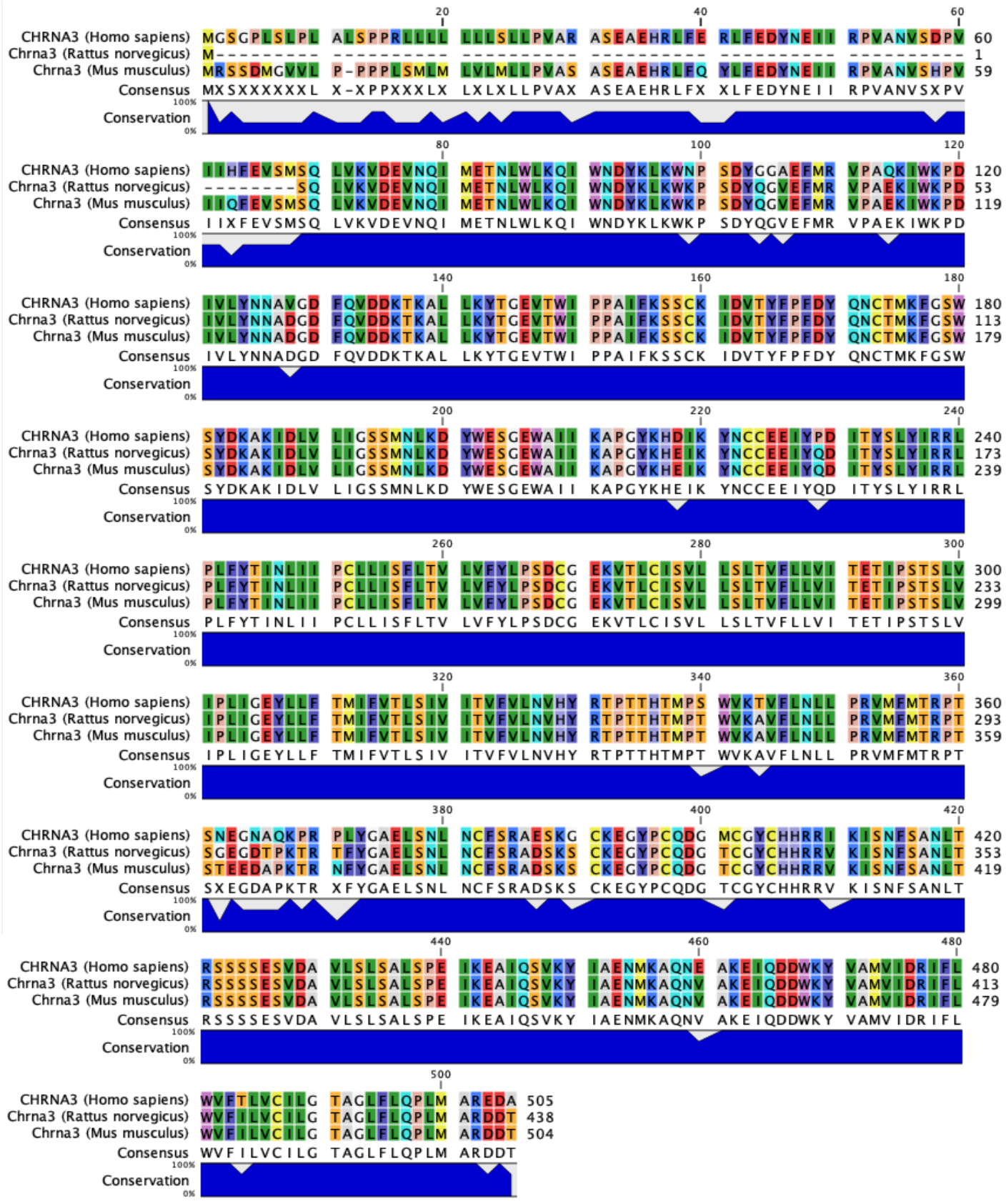
Appendix Figure 11. Muscarinic acetylcholine receptor 5 alignment.



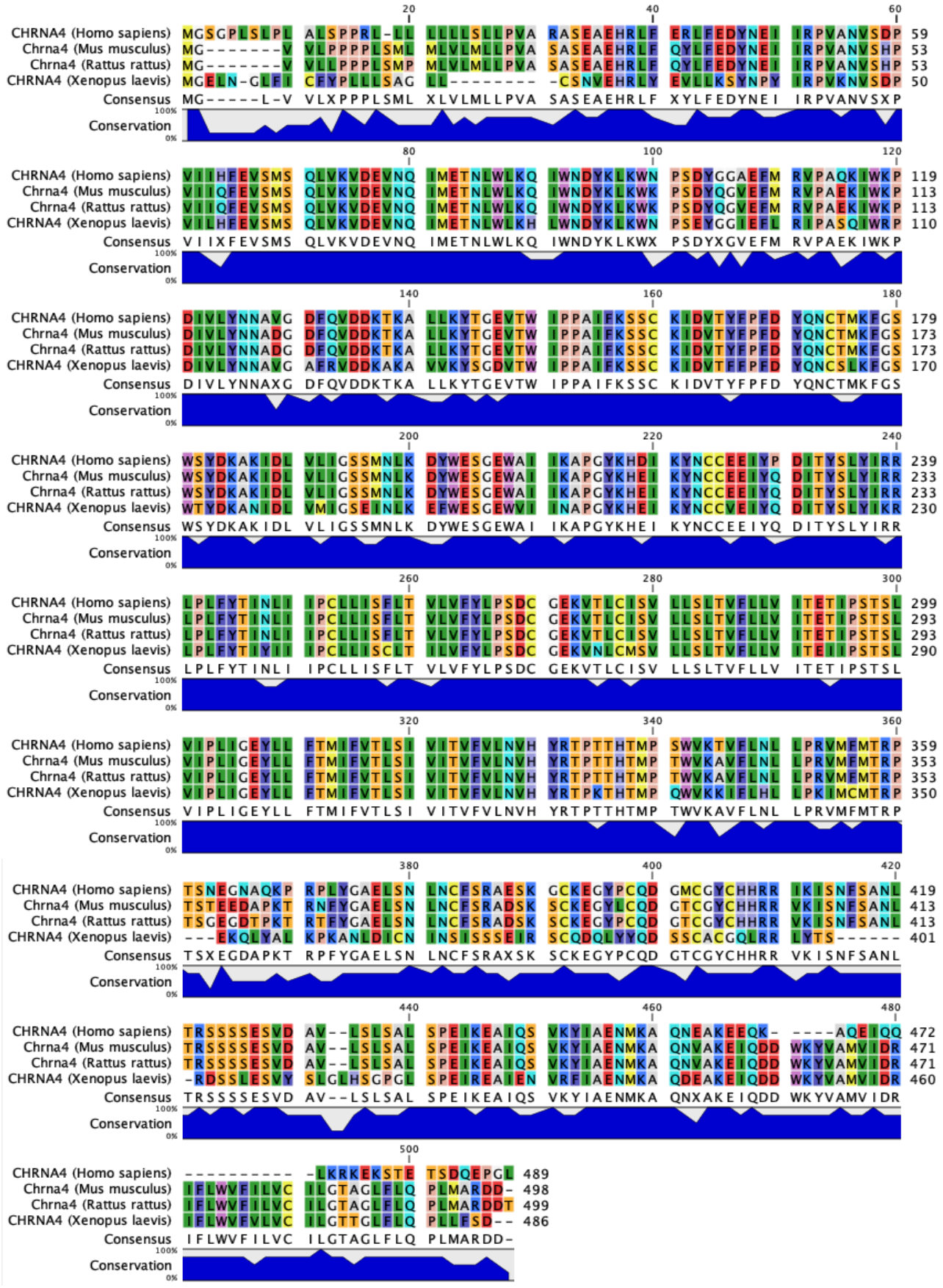
Appendix Figure 12. Nicotinic acetylcholine receptor subunit alpha 1 alignment.



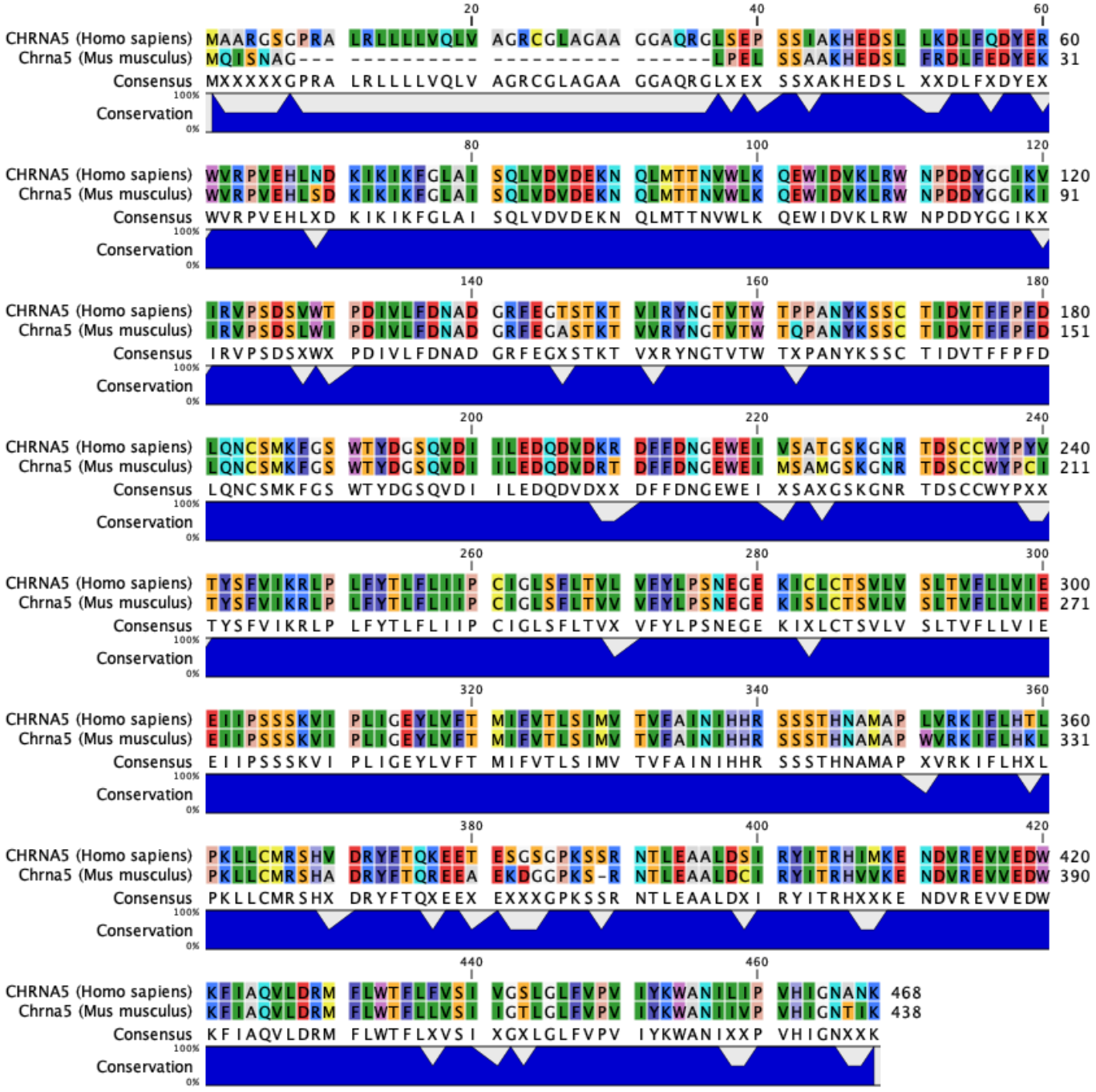
Appendix Figure 13. Nicotinic acetylcholine receptor subunit alpha 2 alignment.



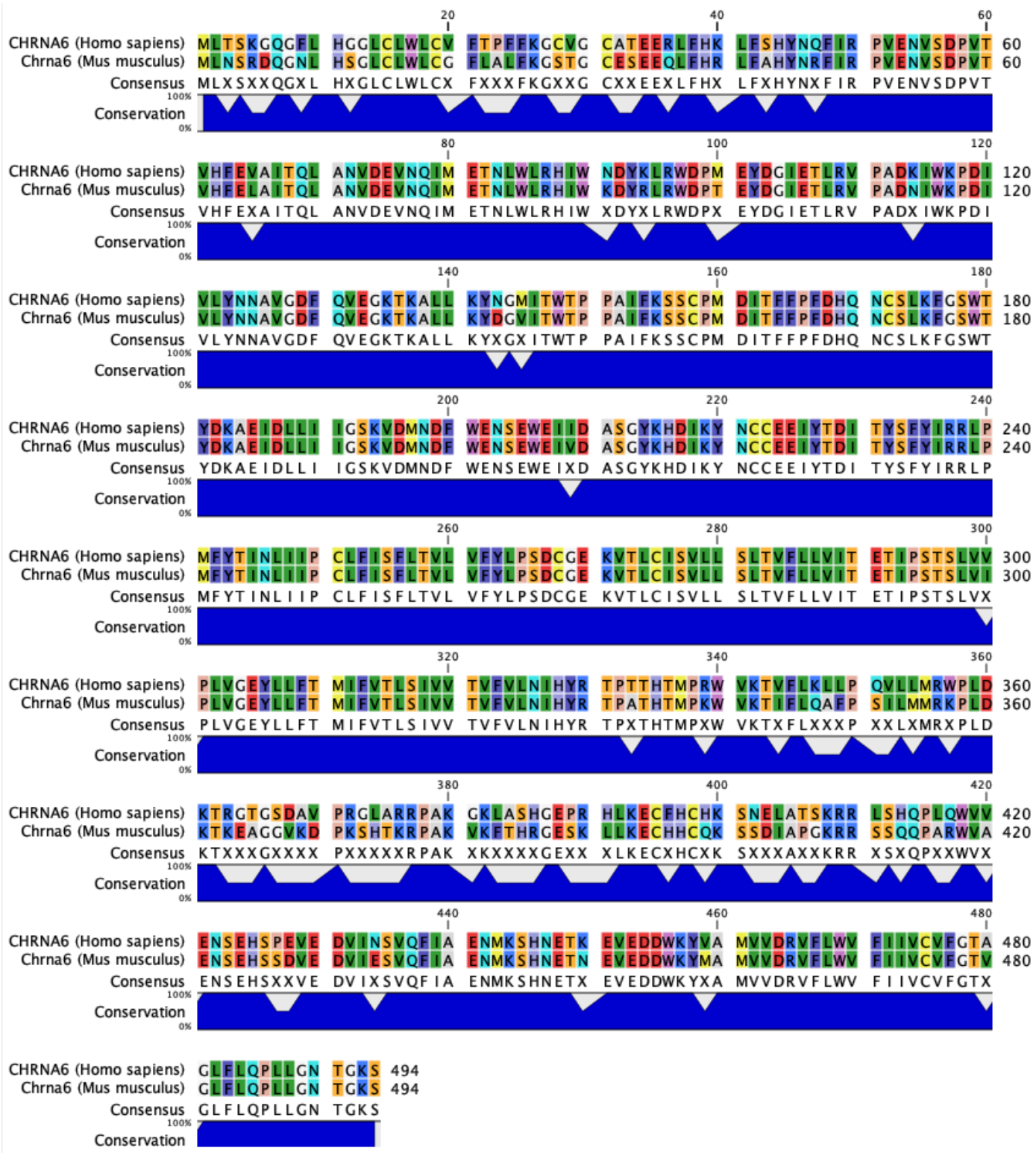
Appendix Figure 14. Nicotinic acetylcholine receptor subunit alpha 3 alignment.



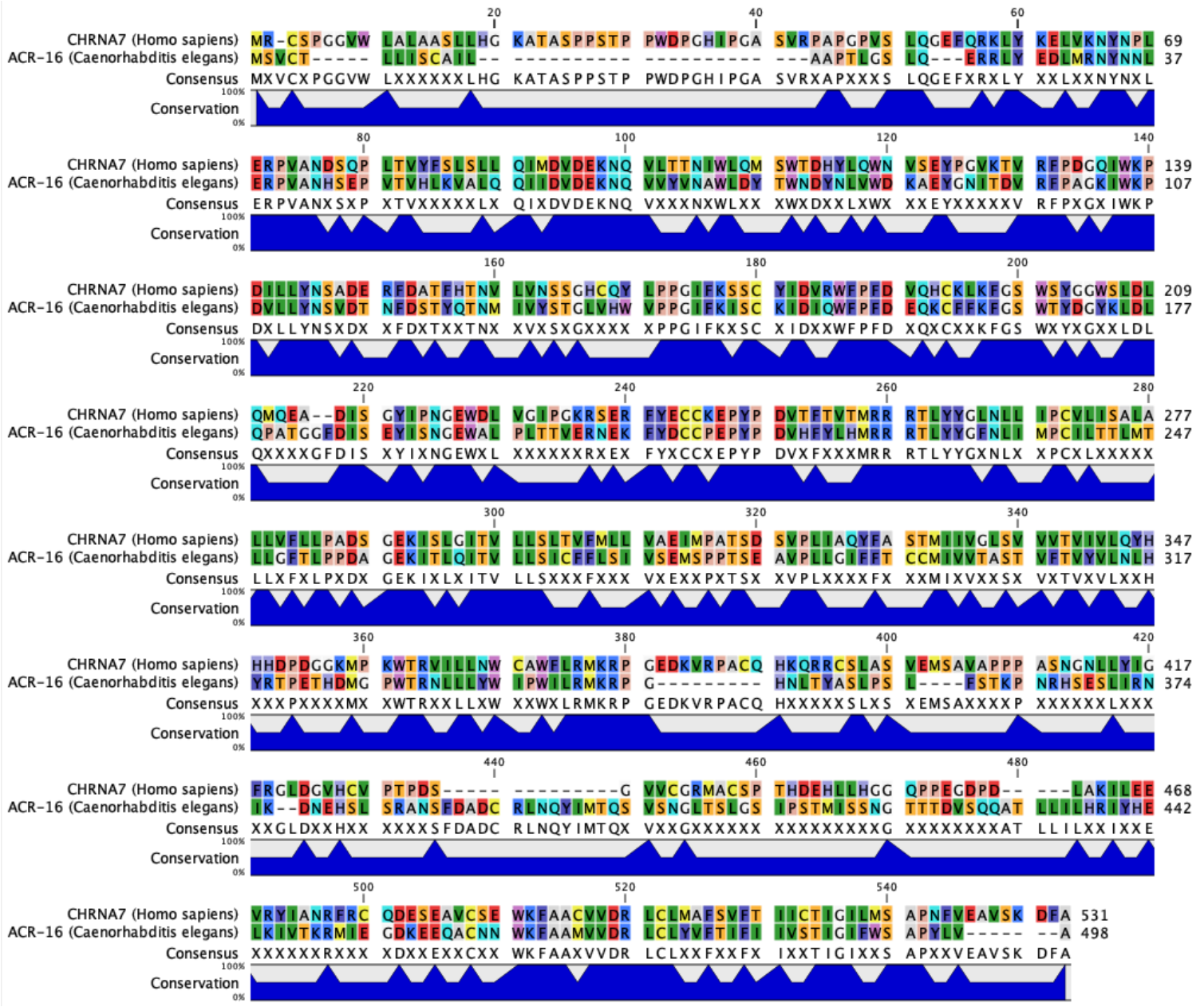
Appendix Figure 15. Nicotinic acetylcholine receptor subunit alpha 4 alignment.



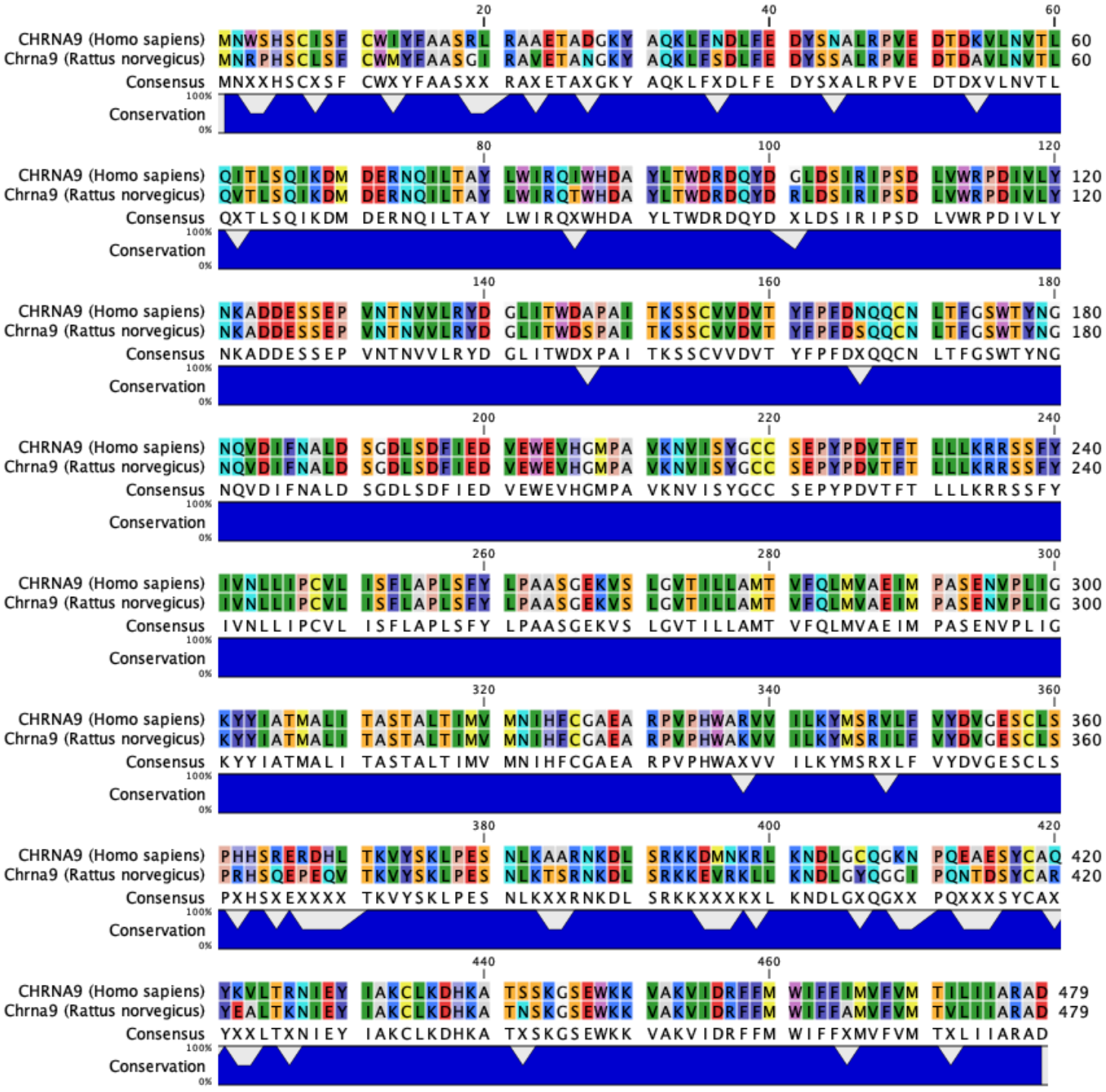
Appendix Figure 16. Nicotinic acetylcholine receptor subunit alpha 5 alignment.



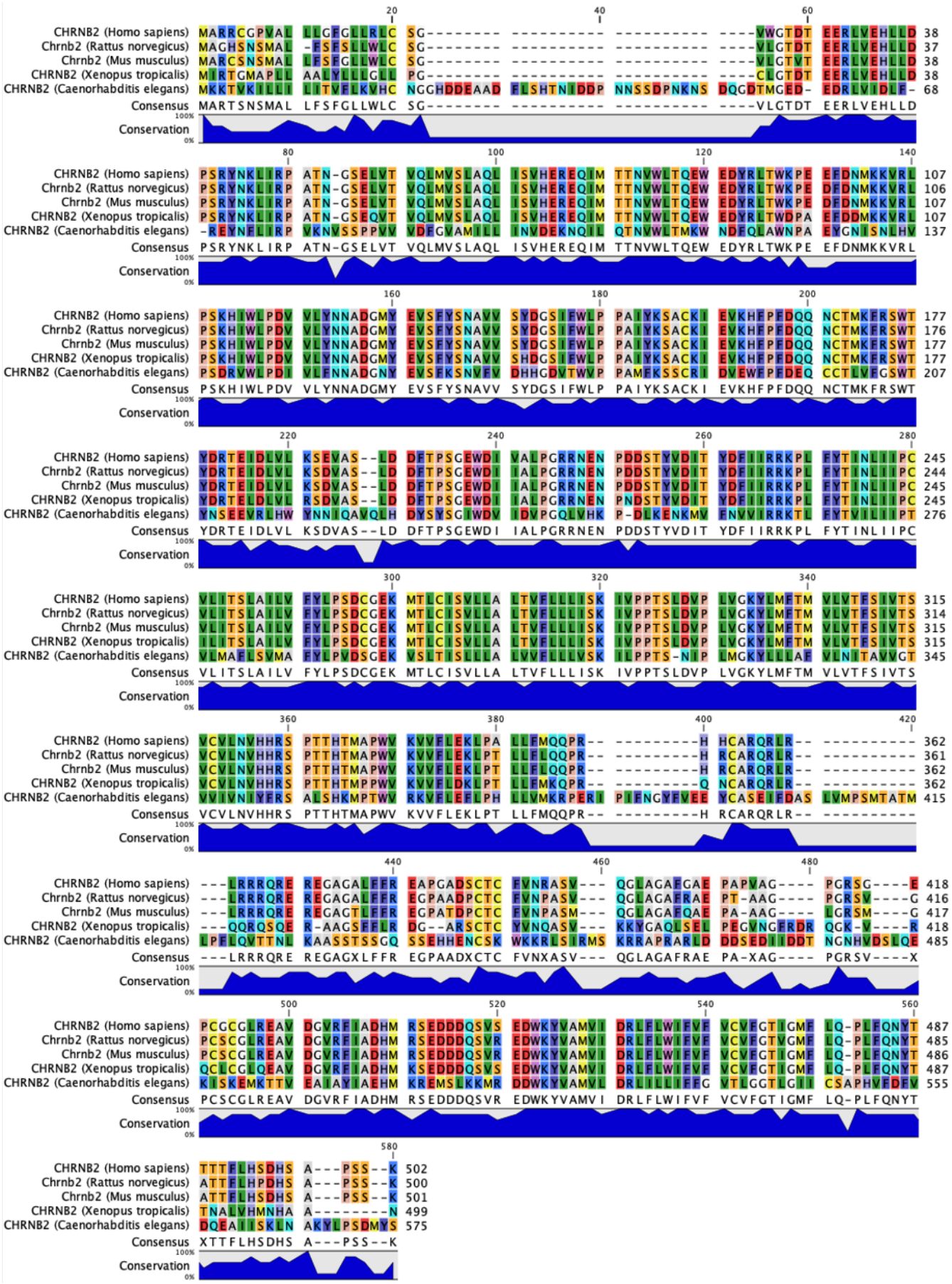
Appendix Figure 17. Nicotinic acetylcholine receptor subunit alpha 6 alignment.



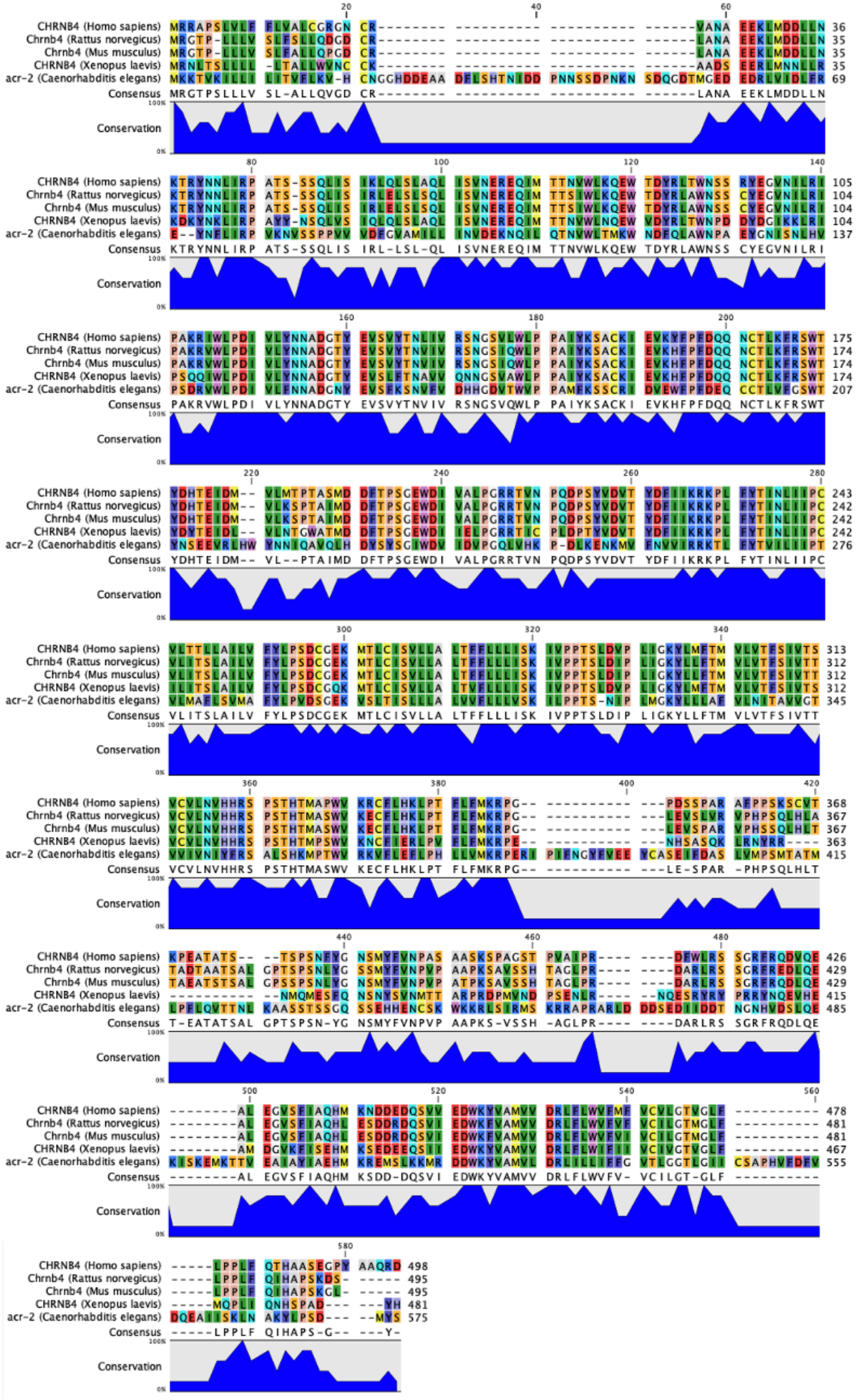
Appendix Figure 18. Nicotinic acetylcholine receptor subunit alpha 7 alignment.



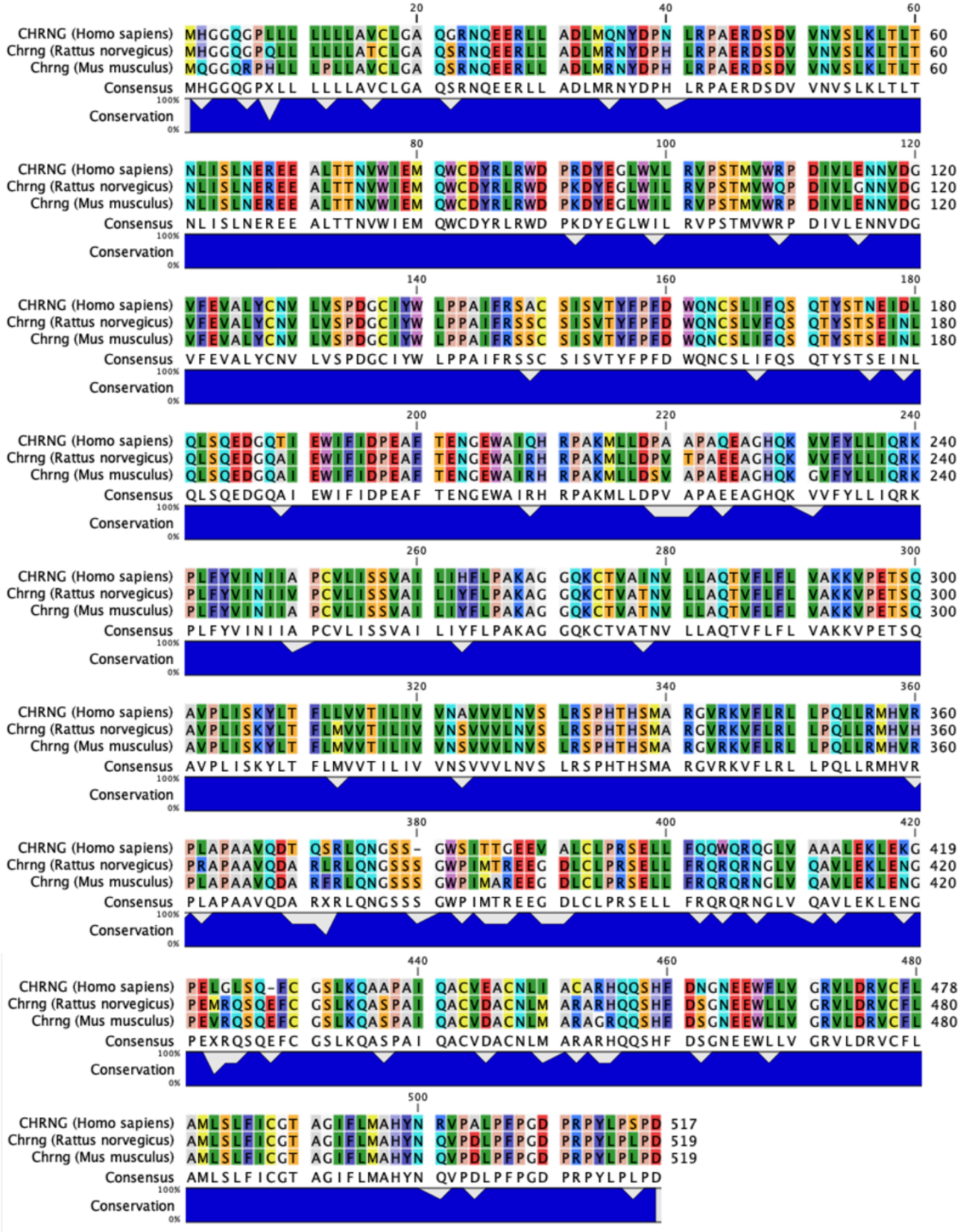
Appendix Figure 19. Nicotinic acetylcholine receptor subunit alpha 9 alignment.



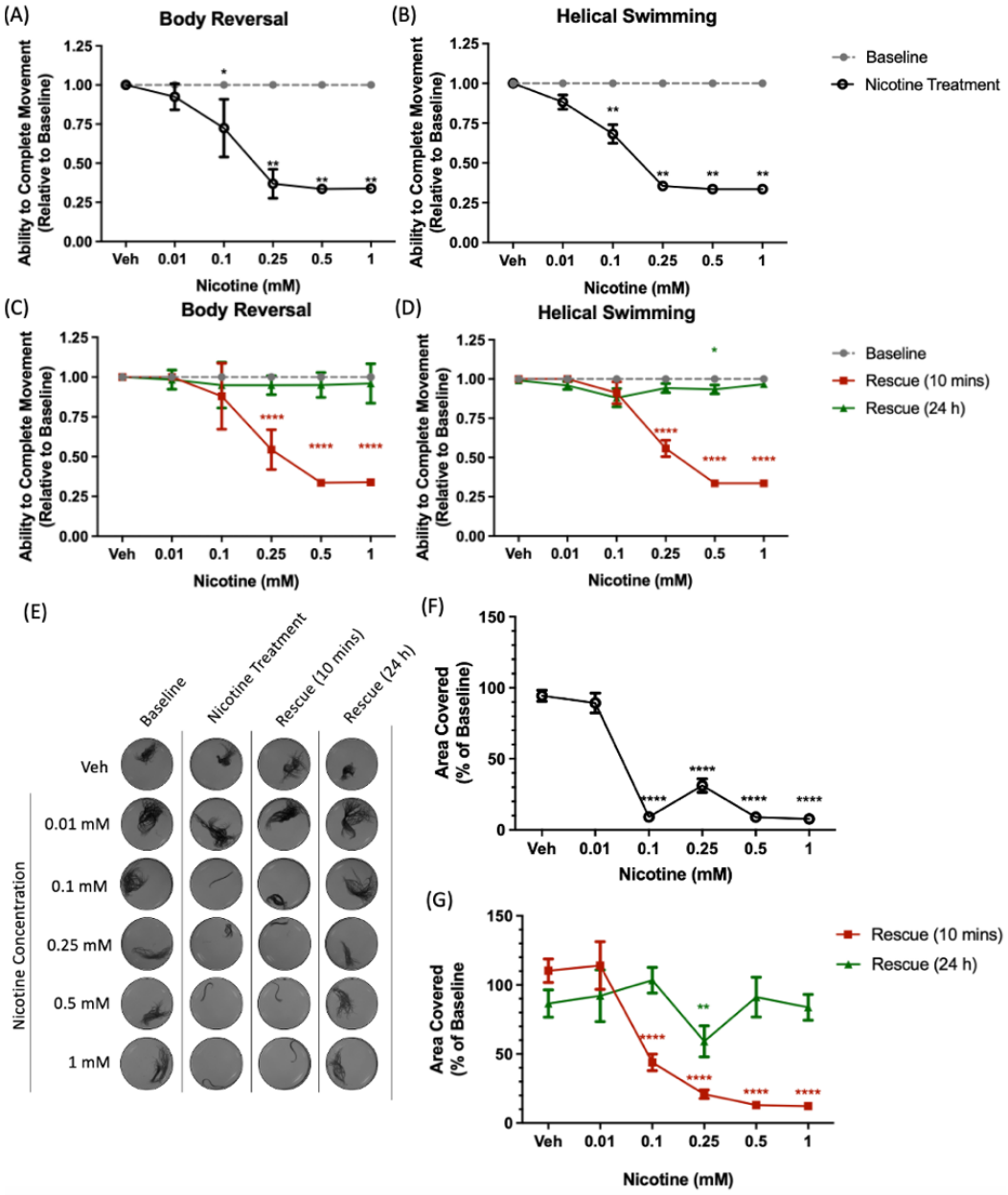
Appendix Figure 20. Nicotinic acetylcholine receptor subunit beta 2 alignment.



Appendix Figure 21. Nicotinic acetylcholine receptor subunit beta 4 alignment.



Appendix Figure 22. Nicotinic acetylcholine receptor subunit gamma alignment.



Appendix Figure 5. The effect of nicotine on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to increasing concentrations of nicotine (0 – 1 mM) and tested for the ability of tactile stimulation to elicit **(A)** body reversal or **(B)** helical swimming. Nicotine 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 nicotine on free locomotion was measured before nicotine exposure (Baseline), after 10 minutes of exposure to 0 – 1 mM nicotine, 10 minutes after nicotine removal (Rescue (10 mins)) and 24 hours after nicotine removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(F**) nicotine treatment and **(G)** removal of nicotine for 10 minutes and 24 hours are the mean. Data is presented as a percentage of the area of baseline movement. Error bars represent the standard error of the mean n=8 for each concentration. Veh: artificial pondwater. *p<0.05, **p<0.01, ****p<0.0001. (Data was generated by Julanta Carriere).

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