UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Centre for Biological Sciences

Ethanol: Response and Mechanisms in *Caenorhabditis elegans*

by

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Thesis for the degree of Master of Philosophy

May 2012

ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

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ETHANOL: RESPONSE AND MECHANISMS IN *CAENORHABDITIS ELEGANS*

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The aim of this project is to identify behavioural phenotypes associated with ethanol in *C. elegans* and potential molecular targets for ethanol that underpin an ethanol response. This will be achieved through a multi-lateral approach using bioinformatics, genetics and neuroscience based methodology.

In accord with previous experiments, behavioural analysis showed that several phenotypes could be used to describe and assess the state of ethanol induced intoxication and dependence over a range of concentrations. *C. elegans* exhibits an inhibition of locomotion at high ethanol concentrations. This manifests as a reduction in the population that chemotax towards a food reward whilst lower ethanol concentrations show no such reduction in population chemotaxis. There is also a change in locomotion which characterises an ethanol withdrawal; this is a separate response from the intoxicating behaviour seen at higher concentrations. Similar to the response seen in populations, individual worms show a dose dependent reduction in pharyngeal pumping rate. This also shows no significant difference at lower concentrations to their untreated counterparts.

Observations from studying pharyngeal pumping indicate that worms do show a behavioural response at lower ethanol concentrations. Worms placed in an environment with food and ethanol will not exhibit feeding behaviour as control worms do, instead worms disperse away from the food source. This behaviour can be observed at a threshold of around 10mM ethanol. It is unclear how ethanol causes this phenotype. Overall, these data provides new paradigms for assessing low dose effects. These assays will be important for future studies designed to model low dose effects.

With respect to higher doses, ethanol is known to activate cellular and physiological pathways that underpin stress. Here, we have investigated whether the unfolded protein response (UPR) is an important mediator of stress induced by ethanol. Our evidence suggests no clear activation of the UPR by ethanol concentrations that exert behavioural effects. In an attempt to pre-empt genetic data, we initiated a database of genes involved in ethanol responses, which were mapped on to *C. elegans* and human homologues. These were used to build an ethanol network, which could be used to refine investigations of ethanol-related genes in *C. elegans*.

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I, **Ben Ient**

declare that the thesis entitled

Ethanol: Response and Mechanisms in *Caenorhabditis elegans*

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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ACKNOWLEDGEMENTS

I would like to acknowledge the Medical Research Council (MRC) and Gerald Kerkut Charitable Trust (GKCT) for kindly funding this work and fostering the environment that encourages scientific thinking and critical investigation. I would also like to thank Southampton University for providing a research environment and a rich working background in which to further my studies. Finally I would like to thank my supervisors and colleagues for the support, encouragement and motivation to continue forward with my work.

ABBREVIATIONS USED

5HT	5-Hydroxy- Tryptophan
ACSS2	Acetyl-coenzyme A synthetase short-chain 2
AMPA recep	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
ANOVA	Analysis of Variance
AP RATS	Alcohol Preferring
ATF	cAMP-dependant transcription factor
ATF6	cAMP-dependant transcription factor 6
ATP	Adenosine Triphosphate
BiP	Binding immunoglobin protein
BK	Big K+ conductance
bZIP	Basic Leucine Zipper Domain
CAK	Calcium Activated Channel
cAMP	cyclic Adenosine Monophosphate
CB	Cannibinoid receptor
CCD	Charge Coupled Device
CGC	*Caenorhabditis* Genetic Centre
CMK	Calcium modulated kinases
CNS	Central Nervous System
CRE	cAMP Response Element
CREB	Cyclic AMP-Responsive element-binding protein
CRT1	Calreticulin
CYP2E	Cytochrome P450 2E
DAMGO	[D-Ala2, N-MePhe4, Gly-ol]-enkephalin
DAT	Dopamine Transporter
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleaic acid

DOR	Delta Opoid Receptor
EC	Endocannibinoids
ECO	Evidence Code Ontology
eIF2alpha	Eukaryotic Translation Initiation Factor 2 subunit alpha
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ERSE	ER Stress Response Element
FAS	Foetal alcohol syndrome
FITC	Fluorescein Isothiocyanate
FMRFamide	Phe-Met-Arg-Phe-NH_2
GABA	Gamma-Aminobutyric acid
GAD	L-glutamic acid decarboxylasae
GAS-1	Growth Arrest Specific Protein
GFAP	Glial Fibrillary Acidic Protein
GFP	Green fluorescent protein
GKCT	Gerald Kerkut Charitable Trust
GLR	Glutamate Receptor
GLR1	Glutamate Receptor 1
GO	Gene Ontology
GOA1	Guanine nucleotide-binding protein G(o) subunit alpha
GPCR	G-Protein Coupled Receptor
GRP78	78kDa Glucose Regulated Protein
GST	Glutathione S-Transferase
GTP	Guanosine triphosphate
HRDL-1	HRD-Like Protein
HSP	Hereditary Spastic Paraplegia
HSP3	Heat Shock Protein 3
HSP4	Heat Shock Protein 4

IRE1alpha	Serine/Threonine Protein Kinase/Endoribonuclease 1 alpha
IRES	Internal Ribosome Entry Sites
IRS1	Insulin Receptor 1
JAMP	JNK Associated protein
KEAP1	Kelch-like ECH-associated Protein 1
KID	Kinase Inducible Domain
KOR	Kappa Opoid Receptor
LB Broth	Lysogeny broth
mAChR	Muscurinic AcetylCholine Receptor
MAP kinase	Mitogen activated protein kinase
MOR	Mu Opoid Receptor
MRC	Medical Research Council
mRNA	Messenger Ribonucleaic Acid
mtUPR	Mitochondrial UPR
MUNC	Mammalian uncoordinated protein homologue
NAD-ADH	Nicotinamide Adenine Dinucleotide - Alcohol dehydrogenase
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
NF-kappaB	Nuclear Factor kappa-light-chain-enhancer of Activated B cells
NF-Y	Nuclear Transcription factor Y
NGM	Nematode Growth Media
NHS	National Health Service
NMDA	N-Methyl-D-aspartic acid
NMR	Nuclear Magnetic Resonance
nor-BNI	Norbinaltorphimine
NPR-1	Natriuretic peptide receptor A/guanylate cyclase A
NPY	Neuropeptide Y
NR-1 subunit	NMDA Receptor 1 subunit

NR-2 subunit	NMDA Receptor 2 subunit
NSF	N-ethylmaleimide-sensitive factor
OD600	Optical Density 600
ORL1	Opoid Receptor-Like 1
PERK	PKRK like ER kinase
PKA	Protein Kinase A
PLC	Phospholipase C
PLP cofactor	Pyridoxal-phosphate cofactor
PMAT	Plasma Membrane Monoamine Transporter
PNS	Peripheral Nervous System
RNAi	Ribonucleaic acid Interference
RNF5/RMA1	
ROI	Region of Interest
ROS	Reactive Oxygen Species
SER-1	Serotonin Receptor 1
SER4	Serotonin Receptor 4
SERT	Sodium-Dependant Serotonin Transporter
SNAP-25	Soluble NSF Attachment Protein
SNARE	SNAP-Receptor
SOCE	Store Operated Calcium Entry
SREBP	Sterol Regulatory Element-binding Protein
SSRIs	Selective Serotonin re-Uptake Inhibitors
STIM1	Stromal interaction Molecule 1
STIM2	Stromal interaction Molecule 2
TUN	Tunicamycin
UBL5	Ubiquitin-like Protein 5
UK	United Kingdom
uORF	Upstream Open Reading Frame

UPR	Unfolded Protein Response
VAMP-2	Vesicle-associated membrane protein 2
XBP1	X-Box Binding Protein

1 Introduction

1.1 Ethanol toxicity, metabolism and distribution

Ethanol is a 2 carbon, aliphatic molecule with a hydroxyl group. It is the active ingredient in alcoholic beverages consumed globally. It comes from the natural product of anaerobic fermentation by the yeast *Saccharomyces cerevisiae* and has been utilised by humans throughout recorded history. Such is the popularity of ethanol as a social beverage that in 2006 the total UK household expenditure on alcohol totalled approximately £41.6bn. This is greater than the entire UK national defence budget for 2010/11. The consumption of alcohol is not risk free and comes with a considerable health risk depending on the duration and amount consumed. In the UK, as of 2004, the cost to the National Health Service (NHS) is estimated to be between £1.4 billion and £1.7 billion a year. The estimated cost of alcohol-related crime and anti-social behaviour estimated to be up to £7.3 billion per year (NHS Information centre, 2008). The health costs also translate into private sector economic losses, with up to an estimated £6.4 billion lost through alcohol related loss of productivity (UK Cabinet Office, 2004). Thus ethanol is a considerable cause of mortality and morbidity with vast economic cost.

Ethanol is a wide-acting, low specificity central nervous system (CNS) depressant. The acute effects of ethanol range from stimulation and euphoria at low doses through to relaxation and sedation at moderate to high doses. At higher doses ethanol acts as an anaesthetic, which can depress the CNS to such an extent as to lead to coma and cessation of respiratory function. Further complicating the risks associated with acute intoxication, chronic consumption can also lead to severe health problems dependent on the drinking pattern. Ethanol has both positive, reward, and negative, relief from, reinforcing affects which can lead to psychological and physiological dependence. Physiological dependence is associated with additional medical complications to those associated with acute and chronic intoxication. Withdrawal from dependence can result in convulsions, coma and death; it is commonly treated by drugs with similar sites of action to alcohol, such as benzodiazepines (Schuckit, 2009).

1.2 Ethanol metabolism

Approximately 95% of ethanol is metabolized by the body, with the remainder passed out in the breath, sweat and urine. In humans, alcohol is metabolized by a rate of 10 g/hour (Zakhari, 2006). Ethanol is metabolised by the enzyme alcohol dehydrogenase, which is predominantly found in the liver but is expressed throughout the body. Ethanol is metabolised into acetaldehyde and then to acetic acid by the enzyme acetaldehyde dehydrogenase. Acetaldehyde is an unstable product and can form free radicals under cellular conditions. Some of the effects of ethanol intoxication and withdrawal are believed to be due to acetaldehyde formation

(D'Addario et al., 2008), although this theory remains controversial due to the low levels of serum acetaldehyde and the difficulty of it crossing the blood brain barrier. Acetic acid can either be directly excreted in the urine or converted to acetyl Co-enzyme A by the enzyme ACSS2, where it can be further metabolised in the Krebs cycle. Ethanol is also metabolized by catalase and cytochrome P450 2E1, although the extent to which these two enzymes play a role *in vivo* is unclear; these enzymes are thought to act when ethanol levels are high or in regions with low alcohol dehydrogenase activity, such as in the central nervous system.

1.3 Ethanol distribution

Ethanol quickly diffuses through the body showing no major localisation at any one organ (Gifford et al., 2008). Molecular interaction simulations and NMR studies show that ethanol resides in the phosphate backbone of the lipid bilayer (Chanda and Bandyopadhyay 2004, Gurtovenko and Anwar 2009). Blood alcohol levels are indicative of brain alcohol levels. The liver is the main organ associated with ethanol metabolism and therefore is considerably damaged after chronic ethanol drinking. Alcoholic fatty liver disease, alcoholic hepatitis and cirrhosis are often the outcomes of chronic ethanol abuse. Away from the liver several organs suffer from the abuse of chronic ethanol consumption. The gastric system is at a higher risk of developing cancer on ethanol abuse. The heart may suffer with alcoholic cardiomyopathy and a higher risk of atherosclerosis. Excessive alcohol consumption is also a risk factor for type 2 diabetes. Fertility is also reduced with chronic ethanol consumption.

In the brain chronic ethanol abuse can result in Wernicke's encephalopathy and Korsakoff's syndrome. Two conditions associated with poor motor coordination, ataxia, short term memory loss and confusion. Sufferers often have overlapping symptoms and illness is sometimes classified as Wernicke-Korsakoff syndrome. The brain may also suffer atrophy of the prefrontal cortex in alcoholics.

Ethanol when ingested contributes to a huge range of health problems dependent on the concentration of ethanol and the length of time it is consumed over. The wide scope of ethanol's actions is apparent in the distribution of health problems over the body. These are underwritten by a large number of molecules that ethanol is thought to interact with. As with many drugs that work to affect the CNS, ethanol controls a varying degree of behaviours. These too are controlled by the many molecules that ethanol interacts with in a time and dose dependent fashion.

1.4 Molecular targets of ethanol in *C. elegans*

1.4.1 SLO-1

SLO-1 is a BK ("Big K^+ conductance") calcium activated potassium channel (CAK) activated by increases in intracellular calcium concentration or membrane depolarisation. The role of

CAKs is as action potential inhibitors through reducing cell excitability via hyperpolarisation. SLO-1 tends to be found at neuron-smooth muscle junctions at both the presynaptic and post synaptic terminals (Holden-Dye et al., 2007). *C. elegans* SLO-1 has been shown to be activated by ethanol between 20-100mM. *slo-1* mutants are resistant to ethanol and *slo-1* gain of function mutants resemble ethanol intoxicated worms (Davies et al. 2003). Ethanol has been shown to inhibit the frequency of body bends, locomotion speed and egg laying, of which locomotion speed and egg laying defects are absent in *slo-1* mutants. Changes in ethanol resistance were independent of membrane permeability and ethanol metabolism. The effects of ethanol resistance were not due to hyperactivity caused by loss of *slo-1* as confirmed by mutant comparison and aldicarb resistance. In general, acute ethanol activates the SLO-1 channel and depresses neuron firing.

Chronic ethanol consumption affects the SLO-1 channel by reducing sensitivity to ethanol, increasing SLO-1 expression and reducing the number of channels at the cell membrane. Alternatively, increased *slo-1* expression has been linked to an increase in positive acting cAMP response element-binding protein isoforms (CREB), inducing expression of *slo-1* on ethanol exposure. These changes may underlie the compensatory changes made in ethanol tolerance as a result of chronic consumption. Underlying these changes may also be a role of membrane lipids and accessory proteins (Yuan et al., 2000). Indeed the membrane lipid raft hypothesis has become a popular theory for describing the action of alcohol on certain membrane components, and there is certainly evidence for a role of lipid and ethanol structural content in the membrane affecting membrane proteins.

1.4.2 GAS-1

GAS-1 is a 49-kDa subunit of complex I of the mitochondrial electron transport chain in *C. elegans*. Mutations in the *gas-1* gene increase sensitivity to ethanol. Complex I is required for the first steps of oxidative phosphorylation; the transfer of an electron from NADH to a quinone. The 49kDa GAS-1 subunit is conserved between species and required for complete complex I subunit assembly and function. Complex I is the mitochondrial electron transport chain component most sensitive to ethanol and volatile anaesthetics. Measuring the levels of oxidative phosphorylation in wild-type and *gas-1* mutants Kayser et al. concluded that sensitivity arose solely from complex I inhibition. Sensitivity of *gas-1* mutants to ethanol is specifically because of the reduced rate of complex I activity (Kayser et al., 2003). The authors suggest that ethanol may affect movement because of the reduced output of ATP. It is also worth noting that the authors raise concerns about the potential difference in mitochondrial diffusion rates and site specific ethanol concentration when extrapolating from in vitro to in vivo. Previous work by the group has also linked *gas-2* and *unc-79* with ethanol sensitivity and locomotion (Morgan and Sedensky, 1995).

1.4.3 NPR-1

npr-1 encodes a putative neuropeptide Y family, GPCR protein which binds the ligands of two FMRFamide-related peptides. *npr-1* has been shown to alter the behaviours of locomotion, social aggregation and food bordering (de Bono et al. 2002, Gloria-Soria and Azevedo 2008, Harvey 2009). The wild *C. elegans* strain CB4856 has a single point mutation, V215F, which results in a lower function allele of which no other isotypes have been found to exist in other natural populations. Ethanol acts as a depressant of locomotion in worms; but has less of an affect over time due to the development of tolerance. The CB4856 strain develops ethanol tolerance more rapidly than N2 independent of initial response, locomotory speed or metabolic clearance. This difference in tolerance maps to the *npr-1* locus (Davies et al., 2004). NPR-1 plays a role in negatively regulating the development of acute tolerance. Evidence suggests that the suppression of food dependent social behaviours by NPR-1 and the suppression of acute ethanol tolerance are separable affects and are likely to work through separate pathways. It is believed that the two ligands of NPR-1, Flp-18 and Flp-21 may play a role in NPR-1 mediated tolerance, although Flp-21 mutants showed no difference from N2 and Flp-18 has yet to be analysed. The evidence suggests that ethanol increases NPR-1 activity, thus resulting in a down regulation of this pathway in a plastic response to exposure, with withdrawal from ethanol then leaving this down regulation exposed to normal conditions (Davies et al., 2004).

1.4.4 Exocytosis and the SNARE complex

Exocytosis of neurotransmitters from the presynaptic nerve requires vesicle fusion and release of pre-stored transmitters. This is achieved via the SNARE complex, which consists of three main proteins SNAP-25, VAMP-2 and syntaxin-1. Munc18-1 is a murine SM family protein that associates with syntaxin-1 to assist in vesicle transport, vesicle docking and syntaxin-1 transport (Deak et al. 2009, Rodkey et al. 2008). Munc18-1 associates with the SNARE complex protein syntaxin-1 and controls sensitivity to ethanol. A D216N mutation in the protein regulates ethanol preference, and appears to modulate SNARE complex binding by Munc18-1. The mutation broadens the duration of single exocytotic events. An orthologous mutation in *C. elegans unc-18* also confers resistance to ethanol (Graham et al., 2009).

There is no obvious defect in the synaptic machinery in the *C. elegans* mutants, although it does seem that the mutation may increase synaptic output. This would possibly indicate that ethanol would work to reduce synaptic transmission and that mutations that affect the exocytotic output may confer resistance to alcohol. This correlates well with evidence that resistance to the acute effects of ethanol is a risk factor for alcoholism. Alterations to the vesicle fusion process and resistance to ethanol have also been recently noted in studies looking at the proteins RAB3 and AEX3, which control vesicle docking and fusion (Kapfhamer et al., 2008). This implicates ethanol in the wider machinery of synaptic output as a mechanism for the acute effects of intoxication and the development of alcoholism.

1.4.5 Heat shock proteins and chaperones

Mutagenesis screens identified four genes that conferred resistance to the anaesthetic affects to ethanol (Hong et al., 2008). These genes were jud-1 to jud-4 and remain poorly characterised. jud-4 is C02D4.1 which shares limited homology with the mammalian Homer proteins. jud-4 is not expressed neuronally but in the hypodermis and vulva muscles. It is not clear how these genes interact with ethanol or the biological system to bring about changes in sensitivity to anaesthetics (Hong et al., 2008). Earlier work by the same group have also looked at a large scale genomics approach to the actions of ethanol, and characterised what appears to be an induction of the unfolded protein response. They characterised genes that may be affected by ethanol exposure at different time points using Microarray analysis.

Of interest was that ethanol induced a lot of heat shock genes and two non-chaperone genes, glr-2 and T28C12.4 a protein with limited homology to human neuroligin. As we see later on GLR may require the unfolded protein response (UPR) equipment for specialised folding, and an upregulation may indicate that transport is perturbed (Shim et al., 2004). The group used an ethanol concentration of 7% v/v in M9 buffer. They found that the 230 non-redundant genes were differentially expressed. They identified a region upstream of several genes involved in the stress response which they named the ESRE (ethanol and stress dependent response element). This sequence was conserved between *C. briggsae* and *C. elegans* (Kwon et al., 2004). It has functional overlap with regulatory elements described by another group (GuhaThakurta et al., 2004). They later went on to show the difference in regulatory sequences of different aspects of the heat shock proteins on various stresses including ethanol (Hong et al., 2004). This evidence offers a large support to the idea that ethanol stress is a trigger for the UPR.

1.4.6 CREB

cAMP response element binding protein (CREB) is a downstream bZIP transcription factor with two isoforms alpha and delta. It has a common KID domain with a serine residue at position 133 which gets phosphorylated. Phosphorylation of serine 133 allows binding of CREB to the CRE region in the promoters of 100+ genes (Suo et al., 2006). It is predominantly associated with activation of upstream metabotropic receptors and changes in calcium or cAMP dependent signalling. It holds a significant interest with relation to this project because of its homology to the ATF proteins activated on the UPR, its role in ethanol mediated neurodegeneration and its possible activation by NPR-1 and the link to ethanol mediated tolerance (Davies et al., 2004).

Glutamate receptor mediated excitotoxicity and oxidative stress are two of the prevalent mechanisms associated with neurodegeneration. The underlying toxicity of these factors is mediated by the antagonistic actions of the transcription factors CREB and NF-κB. These two transcription factors mediate toxicity by antagonistically binding DNA. This can be demonstrated with drugs that either increase CREB binding to DNA, and thus dampen toxicity,

or decrease CREB binding to DNA and increase toxicity. Ethanol decreases CREB levels and increases levels of NF-κB DNA-binding activity, thus increasing neurotoxicity (Zou and Crews, 2006).

Rat based binge drinking models of ethanol consumption result in neurodegeneration in selected brain regions. Both CREB and NPY are diminished in the hippocampal dentate gyrus during binge drinking but increase on ethanol withdrawal. The expression of these two proteins is negatively correlated with neurodegeneration, which is more prevalent after 4 days of binge drinking. Neurogenesis may be controlled by CREB and this is shown to be decreased during binge drinking whilst increasing after sustained abstinence (Pandey et al., 2005a).

Increased anxiety is often seen as a risk factor for the development of alcohol abuse. Withdrawal after chronic exposure decreases phosphorylated (activated) CREB and NPY in different regions. This process in the central amygdala may be linked to ethanol withdrawal mediated anxious behaviour. Administration of a PKA inhibitor into the central amygdala reduces phosphorylated CREB levels and increases anxious behaviour and ethanol preference. CREB levels are lower in preferring rats vs. non-preferring. CREB may mediate anxiety and alcohol preference via NPY modulation (Pandey et al., 2005b). Therefore CREB may act as a genetic determinant of alcohol abuse. CREB has also been shown to help mediate the effects of self-administration of several drugs of abuse. Ethanol increases the levels of phosphorylated CREB in the nucleus accumbens; promoting self-administration. This may be due to an increase in striatial proenkephalin (Newton and Messing, 2006).

In *C. elegans* CREB plays an important role in synaptic plasticity and starvation. The protein CREB maps to the *C .elegans* homolog CRH-1. CRH-1 is activated in a subset of neurones, the SIA neurons, on starvation. This is mediated by the GPCRs *ser-3* and *egl-30*. It is partially dependent on phospholipase C and is negatively regulated by GOA-1 and calcium/calmodulin dependent kinase (CMK-1) (Suo et al., 2006). CRH-1 also controls foraging rate in *C. elegans*. CRH-1 modulates *tph-1* expression in the ADF neurons, whose post-synaptic effects are controlled by the (5-HT2-like) SER-1 receptors. The serotonergic circuit is also contributed to by the interneuron RIH which lacks any expression of *tph-1*. The RIH serotonergic phenotype relies solely on exogenous sources of neurotransmitter, through the ADF neurons. CRH-1 drives expression of *tph-1* which controls 5HT metabolism. 5HT is responsible for negatively regulating foraging rate through SER1 and SER4 (Zubenko et al., 2009). These two results would seem at odds because Suo *et al.* suggest that starvation induces CREB whilst Zubenko *et al.* suggest that this would reduce foraging rate. Clearly the picture is more complicated and needs to be reconciled. But it does suggest a role for *tph-1* and *crh-1* in starvation induced behaviour.

1.4.7 GABA

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter which binds to two sub sets of receptor, ionotropic receptors $GABA_A$ and $GABA_C$ (chloride pore forming) and metabotropic receptors $GABA_B$ (G-protein coupled). GABA is synthesised in the cytoplasm from glutamate by the enzyme L-glutamic acid decarboxylase (GAD) using PLP as a co-factor. The effects of ethanol have been primarily noted in the $GABA_A$ and $GABA_B$ receptors (Federici et al., 2009; Werner et al., 2009). $GABA_A$ receptor is a chloride pore composed of 5 of the 19 available protein isoforms (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ). These combinations would result in a large number of receptor subtypes if all associations were independent, but this is not the case and many of the subunits associate only with others.

Each subunit consists of a large, extracellular, N-terminal, cysteine loop containing domain attached to four transmembrane domains and a short extracellular C-terminus. A combination of the protein subunits 2nd trans-membrane regions associate to make up the lumen of the chloride pore. This central chloride pore is involved in the influx of chloride ions into the cell or resulting in hyperpolarisation. The predominant forms of $GABA_A$ receptor subtypes found in the mammalian brain are the 2α12β2γ2 subtype making up 60%, the 2α22β3γ2 subtype at 15% and the 2α32β3γ2 subtype at 10% (Michels and Moss, 2007). The various other subtypes make up the remaining 15% percent. These subtypes are primarily associated with the pre and post synaptic membranes. The delta subunit associating subtypes are only found peri and extra synaptically.

$GABA_A$ receptors bind benzodiazepines barbiturates, anaesthetics, and neurosteroid. Identified in the 1980's, drugs that stimulate the receptors enhance ethanol's actions, whereas drugs that inhibit the receptors reduce ethanol's affect, confirming ethanol's action at the $GABA_A$ site. Overall ethanol works to activate $GABA_A$ receptors leading to a depression of action signal potential by hyperpolarisation. This is thought to contribute to the major depressive effects of ethanol on the CNS. $GABA_A$ receptor subunit alpha has an identified binding site for ethanol. The site sits at A291 and has a pocket of water surrounding the amino acid. On ethanol binding the receptor conformation changes; opening the pore and allowing chloride entry.

There have been many studies looking at the effects of ethanol on the subunit composition of the most predominant $GABA_A$ receptors; evidence suggests that ethanol may selectively increase activity for this receptor subtype although these studies have used high concentrations of ethanol. There is also a current search for binding sites that operate at low levels of ethanol exposure. There have been mixed reports for the activation of the extra-synaptic $GABA_A$ receptors with a subunit composition of 2α42β2δ (Marutha Ravindran et al., 2007), but controversy remains over the concentrations of ethanol needed to activate the receptor (Lobo and Harris, 2008; Sundstrom-Poromaa et al., 2002). One of the main problems associated with subunit specific studies is the plasticity of the GABA receptor subunits when using knockout

studies. Often there have been reports of subunit compensation in knockout models which may skew findings. The new focus is to analyse role of specific subunits with loss of function mutants. These may reflect a more distinct analysis of function.

1.4.8 Glutamate

Glutamate receptors exist in two forms, ionotropic non-selective cation channels and metabotropic GPCR. Glutamate is the main excitory neurotransmitter in the CNS. Glutamate receptors are found predominantly at the post-synaptic membrane. There are three subtypes of ionotropic receptor; NMDA, kainite and AMPA. These channels are permeable to cations and regulated by a number of ionic and peptide signalling pathways (Mattson, 2008).

The NMDA receptor exists as a two subunit complex with a mandatory NR1 subunit and a NR2 (A-D) subunit. The changes in subunit composition are temporally and spatially regulated. Further isoforms of the two subunit types exist due to differential splicing of transcripts. Both subunits have phosphorylation sites which enhance channel function. Phosphorylation by a number of different kinases has been linked to altered activity and trafficking of subunits. NMDA receptors require the cofactor glycine for its activation. In addition to these Mg^{2+} is a blocker of NMDA receptor activity in a membrane voltage dependent manner (Kloda et al., 2007).

Ethanol inhibits the response of NMDA receptors in varying brain regions and is likely to be subunit dependent. Evidence suggests that there is a direct effect of ethanol on the NMDA receptor, either through a reduced potency of the agonist glycine or through subunit specific interactions (Nagy, 2008; Ridge et al., 2008). Post translational modification of NMDA receptors may also be affected by ethanol. Long term ethanol administration results in an upregulation of NMDA receptors to counter the depressive effects of ethanol (Nagy, 2008), although the precise temporal and subunit specificity of ethanol's actions remain under debate. Ethanol withdrawal leads to hyperactivation of the NMDA receptors and is likely responsible for the convulsive and excitotoxic phenotypes in long term users. The result is that withdrawal from ethanol is potentially harmful and has to be treated with neuroinhibitory drugs such as the benzodiazepines.

1.4.9 Endocannibinoids

Endocannibinoids (EC) are believed to play a role in learning, memory, cognition, appetite and metabolic regulation. Cannibinoid receptors (CB) are found predominantly in the CNS and selective PNS target organs such as the kidneys, lungs, liver and immune system. CB receptors are GPCR and exist as two receptor sub-types, CB1 and CB2. CB1 is mainly found in neural tissues whereas CB2 is mainly associated with the immune system. CB receptors are negatively coupled to the adenyl cyclase system and N and P/Q type calcium channels, whilst positively regulating A-type potassium channels and MAP kinases (Bisogno, 2008).

Ethanol treatment either down regulates or desensitises CB receptors and thier G-protein mediated effect (Rubio et al., 2008). A mechanism of desensitisation is unclear, but the CB GPCR show desensitisation on dual ethanol treatment and agonist mediated constitutive activation, and it is plausible that ethanol may work in this manner. It has also been reported that G-protein levels and activity may be affected as a downstream or paralogous effect of ethanol's actions on CB receptors. Tolerance is therefore linked to an upregulation of endocanniboids and their precursors. Much like with other neurotransmitter systems that are suppressed, withdrawal from ethanol results in a sensitisation of the CB system.

It has been suggested that a lower function of CB receptors to ethanol may result in predisposition to alcohol preference (Vinod et al., 2008). Pre-treatment with SR141716A (Rimonabant), an inverse agonist of the CB1 receptor, has been found to decrease consumption and preference of ethanol (Dyr et al., 2008). Antagonism of CB1 reduces the motivation to drink ethanol and CB1 agonists facilitate ethanol consumption. CB1 deletion reduces preference to consume ethanol. It is likely then that CB receptors work to increase alcohol drinking and preference, and may play a role in the development to alcoholism (Malinen and Hyytia, 2008).

1.4.10 Dopamine

3, 4-dihydroxyphenylethylamine (dopamine) is a neurotransmitter and hormone, with 5 receptor subtypes each with their respective variations. Dopamine is synthesised in neurons where it is packed into vesicles for calcium mediated release into the synaptic cleft. D1 type receptors are D1 and D5, and activate adenyl cyclase through Gs G-protein. The D2 type receptors are D2, D3 & D4. The DAT is a dopamine transporter required for reuptake of dopamine into the pre synaptic node. It is what mediates signal strength and duration. DAT is unique to DA neurons and is found in the perisynaptic membrane rather than the cleft. Its action is sodium, potassium and temperature dependent, and is a target for a number of drugs, including cocaine (Foll et al., 2009; Zhu and Reith, 2008). Dopamine is also a precursor to noradrenaline and adrenaline.

Dopaminergic neurons are primarily present in the ventral tegmental area, the substantia nigra and arcuate nucleus of the hypothalamus. Dopamine primarily functions in reward, cognition, motivation and motor activity. It also has a role in pleasure, sexual interaction, salience, pain, sociability and psychosis. The most common attribute associated to dopamine function is in reward prediction error. Dopaminergic neurons are active at the receipt of a novel reward, and their activity dampened when no reward is produced. It is said that the affect mediates the feeling of wanting, and plays a major role in motivation. This is known as the mesolimbic reward pathway.

Drug abuse stimulates release of dopamine from the nucleus accumbens and this is reduced on withdrawal; thus highlighting dopamine as one of the main pathways for reward and reinforcement of drug abuse (Foll et al., 2009). Changes in the dopamine system have been

noted in animal and human studies; with reduced densities of dopamine transporters and D2 receptors in alcoholics. Current work shows that the nucleus accumbens is a filter through which the effects of the dopamine system are mediated, controlling the link between desire and action.

Ethanol has been shown to stimulate the release of dopamine from the nucleus accumbens in a dose dependent manner, by stimulating release rather than by inhibiting DAT. This is likely to be controlled by the ventral tegmental area (Wanat et al., 2009). It is also possible that ethanol's action on other neurotransmitter systems leads to the increased release of dopamine. Withdrawal from ethanol promotes a reduction in dopamine release, and relief from withdrawal promotes dopamine release. The return of dopamine to basal levels after a continual exposure is in line with a tolerance affect. It has been suggested that the levels of basal dopamine and the release of dopamine on ethanol exposure are likely to mediate the differences in non-preferring vs. preferring rodent models. Alcohol preference in rats has been associated with lower B_{max} of D2 receptors, with unaltered binding preference in dopaminergic regions of the brain, whilst studies on D1 and D3 receptors remain inconclusive.

1.4.11 Serotonin (5HT)

5-hydroxytryptamine (serotonin) is a neurotransmitter involved in the modulation of anger, aggression,body temperature, mood, sleep, human sexuality and appetite. In addition to the CNS, serotonin is expressed in the gut, where it mediates cell to cell interactions. The main region of serotonergic neurons is in the brainstem's raphe nuclei, with the axons projecting into the majority of brain areas. There are 7 serotonin receptors (1, 2, 3, 4, 5A, 6, 7) with receptors 5HT1 and 5HT2 having 5 and 3 identified isoforms respectively. All of the receptors are metabotropic with the exception of 5HT3 which is ionotropic. 5HT receptors can modulate the effects of other neurotransmitter release such as glutamate.

Serotonin is removed from the synaptic cleft by uptake through a monoamine transporter, either SERT or PMAT. It is these receptors that are implicated in the effects of cocaine. They are also targets for SSRIs (selective serotonin reuptake inhibitors) which are used to treat anxiety and mood related disorders. This is of interest because around 40% of alcoholics have anxiety related problems or have had prior history of anxiety related problems. It may indicate that levels of serotonin become altered over time in relation to alcohol abuse, or that genetic variation at the serotonin system level may be a risk factor for developing alcoholism (Caldwell and Miczek, 2008).

Application of serotonin receptor antagonists reduce ethanol intake, whereas the application of exogenous serotonin increase the effects associated with ethanol. GPCR including 5HT receptors have their high affinity state inhibited by ethanol (Seeman and Kapur, 2003). Ethanol increases neuronal firing in the ventral tegmental area and decreases firing in Purkinje neurons

with both these affects being enhanced by serotonin application. These affects are dependent on age, with aged rats not showing as significant affect (Jeng et al., 2000). The prevalence of alcohol use with age may have an effect on memory and learning; these may be mediated by serotonin. Oliveira-Silva *et al.* found that ethanol does not have any effect on age related memory and learning impairment and there is no change in 5HT levels related to age. This affect was independent of age extension via calorie restriction (Oliveira-Silva et al., 2007).

Whilst it seems that the use of alcohol does not interact with the serotonin system in older models, the use of alcohol by pregnant women has been shown to have a significant affect. Ethanol exposure during pregnancy is associated with a variety of physical and neurological dysfunctions, giving rise to a spectrum disorder of foetal alcohol syndrome (FAS). Serotonin is vital for the development of the central nervous system and its altered role in development has been linked to ethanol consumption during pregnancy. The role of serotonin in development seems to be controlled through the 5HT1A receptor, which amongst other things causes the release of S-100β. S-100β is a growth factor with multiple outputs. Ethanol treatment increases serotonergic cells in the striatum and hippocampus in rat foetuses. There is also an increase in the area of GFAP staining, an astroglial cell marker, in the striatum and hippocampus, both areas affected in FAS. This links ethanol exposure to perturbed serotonin system which controls CNS development (Ramos et al., 2002).

Serotonin is thought to be responsible for impulsivity and aggression. Alcohol also increases aggression in some individuals and is thought to be a major role in crime, with more than half of rapes, murders and related violence having the offender under the influence of alcohol (Krug et al., 2002). Projections in the prefrontal cortex are believed to regulate alcohol mediated aggression. The SSRI Citalopram has been shown to reduce anxiety and aggressive behaviours associated with ethanol. In mice Citalopram has no effect on alcohol mediated aggression in the first 2 weeks of administration, but after 17 days abolished alcohol mediated aggression whilst not affecting the baseline (Caldwell and Miczek, 2008). Chronic ethanol use is associated with long term changes including neuronal damage. Chronic ethanol consumption is correlated with neurodegeneration in the CA1 region of the hippocampus and reduced 5HTT staining, implicating the serotonin system (Tagliaferro et al., 2002).

1.4.12 Opioid system

The opioid system comprises of five main classes of endogenous opioid neurotransmitters; dynorphins, enkephalins, endorphins, endomorphins and nociceptin. There are three main classes of opioid receptor μ, δ, κ (MOR, DOR, and KOR), yet more remain but are under characterised ζ, ε (zeta, epsilon). In addition there is the ORL1 receptor for nociceptin. All the receptors are GPCR and are found primarily on GABAergic neurons. They are not limited to the CNS, they are also found around the digestive tract.

The central amygdala is involved in mediating stress, fear and anxiety behaviours and is involved in the stress related behaviours of drinking, and possibly the reinforcing affects. MOR receptors and enkephalin are found in the GABAergic neurons of the central amygdala. The link between ethanol reinforcement and MOR is well established, although the mechanisms remain under debate (Kitanaka et al., 2008; Vukojevic et al., 2008), and knockout mice show reduced ethanol consumption and anxiety. MOR antagonists reduce ethanol consumption, and activation of DOR by agonists reduces ethanol consumption (Margolis et al., 2008; Nielsen et al., 2008). KO of the delta receptor show increased ethanol consumption. This highlights the role of MOR as a positively acting regulator of ethanol consumption and DOR as a negative regulator of ethanol consumption.

Ethanol activates MOR in the central amygdala which decreases presynaptic transmission of glutamate and GABA. The decrease in GABAergic neurotransmission may also be dependent on corticotropin-releasing factor (CRF) (Kang-Park et al., 2009). The ventral tegmental area is a nucleus of dopaminergic neurons that is inhibited via GABAergic neurons. These GABA currents can be modulated by ethanol and MOR. Ethanol affects the presynaptic release of GABA and not the postsynaptic GABA receptors. DAMGO, a MOR agonist, and ethanol both inhibit GABAergic neurons. Both reduce GABA transmission and result in exciting dopaminergic neurons. MOR antagonists and competitive agonists reduce excitory effect on dopaminergic neurons (Xiao and Ye, 2008).

Whilst the role of MOR and ethanol is to stimulate reward through activation of dopaminergic neurons, it appears that agonists of KOR inhibit the rewarding effects of ethanol. Both naltrexone and nalmefene, opioid receptor antagonists, reduce ethanol consumption in rats, with nalmefene decreasing the ethanol consumption in AP rats more than naltrexone. This is suggested to be because that nalmefene has a 2 fold higher binding affinity for KOR than naltrexone. Administration of nor-BNI, a KOR specific agonist, to dependent and non-dependent rats reduced ethanol self-administration (Walker and Koob, 2008). This highlights the role of KOR in depression of alcohol consumption.

1.4.13 mAChRs

Muscarinic acetyl choline receptors have 5 receptor subtypes (m1-m5) all of which are G-protein coupled receptors. M1, M3 and M5 receptors are positively coupled to PLC by Gq protein, whilst M2 and M4 receptors are coupled negatively to adenyl cyclase by Gi protein. M1 receptors are expressed in all major brain areas while M2 are limited to the forebrain as a presynaptic auto-receptor that works to inhibit neurotransmitter release. M3 receptors are expressed mainly in the smooth muscle. The M4 receptor is mainly expressed in the striatum and colocalised with dopamine D1 receptors, but is not limited to this area. The M5 receptors are expressed in the substantia nigra and ventral tegmental area. The expression pattern of M4

and M5 receptors suggests a role in drug reinforcement, although there is little evidence for a role in these behaviours.

mAChR antagonists increase locomotory activity on ethanol treatment and this affect is independent from dopamine. Treatment with scopolamine, a non-specific receptor mAChR antagonist, produced locomotory activity increases in ethanol treated FAST and DBA/2J mice greater than either drug alone. Treatment with D2 and D1 receptor antagonists was unable to stop this affect (Scibelli and Phillips, 2009). Ethanol has also been found to be a potent inhibitor of muscarinic receptor-stimulated proliferation of astroglial cells, with an IC_{50} of 10–25 mM.

Activation of M3 receptors by carbachol causes this proliferation by downstream activation of DNA synthesis, and this affect is inhibited by ethanol possibly by interfering with activation of the downstream targets PKCζ and p70S6K (Guizzetti and Costa, 2002). Acetylcholine is associated with learning and memory consolidation, and changes in mAChRs seem to correlate well with ethanol induced deficits in memory and learning. Previous studies point to an increase of mAChRs on the cell surface in SH-SY5Y cells treated chronically with ethanol (Caron and Alling, 2001). This upregulation of mAChRs seems to be a functional response to ethanol treatment inhibiting mAChRs. We also note that ethanol causes an upregulation in M4 mRNA, but not M1 in NG108-15 and NCB-20 cells (Fukamauchi et al., 1998). It is unclear as of yet what these functional changes in mAChR regulation are responsible for in ethanol related behaviour, but it may play a role in acute ethanol stimulation on locomotory activity.

1.5 Behavioural effects of ethanol in *C. elegans*

Whilst several molecular targets for ethanol have been identified above they only offer a brief glimpse into the behaviour of *C. elegans* on ethanol treatment. As the response to ethanol is context and dose dependent it is important to distinctly identify the behaviours associated with each state, from acute treatment through to chronic treatment and the plastic changes that underlie further behavioural changes. It is by classifying these behavioural states as distinct and paradigm-dependent that we are able to build a platform from which to investigate the molecular basis of ethanol activity. Difficulty has always arisen in trying to classify a spectrum of behavioural changes into distinct states, but it is essential for clarification in experimentation and understanding. Here we aimed to identify behavioural states associated with acute intoxication, treatment withdrawal, withdrawal relief and chronic tolerance. Vital to understanding how alcohol works at a molecular level is a need to control the dose of ethanol looked at; further investigation into lower sub-intoxication doses and behaviour are also investigated.

Primarily focus turns to previously developed assays for measuring the dose dependent effect of ethanol with the study of worm locomotion and chemotaxis. Locomotion is defined by a stretch sensitive control of sinusoidal body movement over a substrate, starting with a directed head

movement. It works to propel the worm over an environment. It is also regulated by several over-riding behaviours which work to control direction and speed, such as escape from a nocioceptive response or towards a chemo-attractant. Chemotaxis; the movement of the worm towards a chemical stimuli can also be used as a measure of behaviour. Ethanol as a depressant works to inhibit locomotion and therefore inhibits chemotaxis. This can be used to assess the behaviour of worms on varying ethanol treatment paradigms.

Pharyngeal pumping is vital to worm function; it regulates the passing of bacterial foodstuffs into the gut passage. It can also be used as a behavioural measure of ethanol intoxication. The pharynx is a somewhat distinct microcircuit of neurons regulated by sensory inputs of food availability. Food activates pharyngeal pumping and withdrawal from food suppresses pumping. The pharynx can be used as an estimate of how pharmacological agents such as ethanol can regulate a semi isolated neuronal system and a simple behavioural output.

2 Ethanol Mediated Behaviour

2.1 Introduction

Ethanol is a major source of morbidity and mortality, responsible for 2.5 million deaths annually worldwide. Understanding the mechanism of alcohol intoxication and addiction is of major medical value. To study ethanol in a behavioural and physiological context we can utilise the nematode worm *Caenorhabditis elegans* as an experimental system (See 1.4 and 1.5). *C. elegans*, much like other organisms, has distinct behavioural phenotypes dependent on the concentration of ethanol and treatment paradigm. Acute and chronic intoxication, withdrawal, relief from withdrawal and tolerance are all shown as separate behavioural phenotypes. These behaviours are underpinned by distinct molecular effectors that act in concert to regulate the overall physiological response. By identifying the components it should help to understand the possible mechanisms of ethanol response in higher organisms and how behaviour is controlled at a molecular level.

2.1.1 Ethanol toxicity and response in *C. elegans*

C. elegans is under-utilised for dissecting the pharmacological action of ethanol compared to other experimental models. Nevertheless, *C. elegans* represents a useful model for ethanol experimentation and has contributed to understanding the molecular basis of ethanol response. Using *C. elegans* to model different behavioural states allows the investigation of the underlying regulation. This requires *C. elegans* to show acute responses to ethanol and behavioural plasticity to chronic treatment. This is explored further below.

2.1.2 Locomotion in *C. elegans*

Locomotion is defined as the power or ability to move. In *C. elegans* it is characterised by an undulatory wave that acts to propel the organism forward. Locomotion begins with a controlled directional movement of the worms head; this in turn causes stretch receptors directly posterior of the head to detect a change in tension in the cuticle/surface of the worm. The components that make up the nervous system and muscular controls utilised in worm locomotion are often homologous to components in the mammalian and human nervous system, although not always in directly analogous roles. It is these networks and molecular machinery which are affected by ethanol intoxication, thus by studying locomotion in *C. elegans* we can gain an insight into the possible mechanisms that control human behaviour.

2.1.2.1 Chemotaxis assays and quantitative measure of ethanol effects

Locomotion towards a cue as a result of directed behaviour is called chemotaxis. In *C. elegans* the cue is often a chemical substance associated with the presence of bacterial food. Worms will

chemotax towards gradients of ions, cyclic nucleotides, organic molecules and bacterial species. Movement towards a cue is controlled in a similar way to the random tumble movement exhibited by bacteria. Worms will move up a gradient of an attractant, comparing temporal changes in gradient concentration and deceasing turn frequency on movement towards source, whilst increasing turn frequency on movement away from a food source. This behaviour results in a biased random 'walk' towards a cue.

The presence of food in *C. elegans* suppresses turning behaviour and slows locomotion; maximising time spent in the presence of a food source (Maricq et al., 1995). This behaviour is switched off in when the worm is no longer in a food rich environment. Behaviour changes to a temporally controlled search mode. The initial response, from 0 to 15 min, is to switch to local area search. Turn frequency is increased as is the omega turn count. Speed increases from feeding levels and the angle of each turn increases. The result is a restricted search pattern to the immediate area around the worms starting position. This reduces chance of missing food sources that lie in the immediate vicinity. If food is not found after 15-20 min the worms then switch to long distance search behaviour. Turn frequency, angle and omega turn frequency are decreased whilst speed increases slightly. This has the effect of a more long distance movement away from the starting position to ever distant food sources. This behaviour switch is made after a local search for foods sources.

2.1.3 Pumping behaviour in *C. elegans*

C. elegans will often chemotax towards cues indicative of a food source. Feeding in *C. elegans* is regulated by pharyngeal pumping. The pharynx is the lumen, musculature and neuronal network that sits in the head of the worm at the entry to the gut cavity. The pharynx acts as a pump that draws in bacterial food during feeding. It is regulated by a small number of neurons (twenty) that are connected to the rest of the central nervous system by a single direct neuronal connection mediated by RIP. Thus the microcircuit that regulates pumping is unique in that it is almost completely isolated from other inputs. In addition the pharynx and its distinct microcircuit can be dissected from the worm and studied without the worm body. Again the circuit provides a useful tool in which to study the regulation of an almost isolated system of a small number of neurons which uses the same components as a more complex system in higher organisms.

2.1.4 Ethanol concentrations in *C. elegans* & Humans

Alcohol levels between humans and *C. elegans* are not directly comparable. Levels able to cause acute and chronic intoxication in *C. elegans*, are fatally toxic to humans by many magnitudes. Human blood alcohol content (BAC) of 0.10% by volume, (22mM) just over the drink drive limit in the UK, and enough to cause impairment of motor function and changes to behaviour. *C. elegans* however does not show any impairment of motor function until around

150mM, and severe impairment at 300mM after prolonged exposure. It is unknown why these differences in exposure concentrations are so great. It can be speculated that *C. elegans* has adapted to an environment rich in alcohol, such as rotting fruit, and evolved some resilience to exposure. Others have suggested that the cuticle may provide an entry barrier to ethanol diffusion, and that levels measured suggest a lower more analogous concentration exists internally in the worm (Davies and McIntire, 2004). Experimentation has shown that the lipid cuticle provides little barrier to ethanol and that behaviours present on ethanol exposure are not dependent on a cuticle, such as pharyngeal pumping (Mitchell et al., 2007).

The concentration of ethanol used to test *C. elegans* is one to illicit behaviours that are partially analogous to the behaviours seen in other organisms such as humans and mice, and not directly linked with BAC or exposure concentrations. Whilst slightly limiting, *C. elegans* still provides a strong platform for an ethanol led investigation into the molecular basis of behaviour.

2.2 Methods

2.2.1 Media

2.2.1.1 NGM

Nematode growth media; used as a substrate for worm culture and experimentation. For 5 litres:

NaCl	12g
Peptone	10g
Agar	80g
H_2O	3888ml

Put a large magnetic stirrer bar in a 5l bottle, autoclave, cool to hand hot (~55° C) stirring continuously for about 1 to 1.5hrs and then add:

$CaCl_2$ (1M)	4ml (autoclaved)
$MgSO_4$ (1M)	4ml (autoclaved)
K^+ Phosphate buffer (pH6)	100ml (autoclaved)
Cholesterol (5mg/ml)	4ml (stored at -20˚C)

A Peristaltic pump is used to aliquot 10ml NGM into 55mm Petri dishes. Tubing for the pump wrapped in aluminium foil must also be autoclaved.

To reduce contamination, the area of bench used for plate pouring is swabbed down with 70% ethanol, and gloves are used for the entire procedure.

NGM plates are left to 'dry' (with the lids on) at 20°C for 24 - 48hours before seeding with OP50.

Plates destined for use in ethanol experimentation are devoid of cholesterol, to eliminate any residual effects from the ethanol used to store the cholesterol remaining in the media.

2.2.1.2 Potassium Phosphate Solution

Potassium Phosphate; used as a pH buffer in nematode growth media. For 1 litre:

K_2HPO_4	23g (0.132M)
KH_2PO_4	118.12g (0.868M)
H_2O	to 1L

Autoclaved

2.2.1.3 *E. coli* OP50 cultures

LB agar plates streaked with OP50 are incubated at 37° C overnight, then sealed with parafilm and stored at 4° C.

A fresh plate is streaked with OP50 every month.

Individual colonies of OP50 are grown overnight in 10ml of LB broth in sterile universal bottles, on a shaker at 37°C. 50µl of this bacterial suspension is pipetted at the centre of each NGM plate (without spreading). Seeded plates can be stored at room temperature (20°C) for up to 2 weeks.

2.2.1.4 LB Broth

LB Broth is used as a nutrient source for liquid cultures of *E. coli* OP50. For 1 litre:

Bacto-tryptone	10g
Yeast extract	5g
NaCl	10g

Added the to 800ml H_2O

Adjust pH to 7.5 with NaOH.

Adjust volume to 1L with dH_2O

2.2.1.5 M9 Buffer

M9 buffer is used to wash worms in preparation for experimentation. For 1 litre of M9 Buffer:

KH_2PO_4	3g
Na_2HPO_4	6g

NaCl	5g
$MgSO_4$ (1M)	1ml
H_2O	to 1L
Autoclaved	

2.2.2 Nematode culture

2.2.2.1 *Escherichia coli* OP50 culture.

E. coli OP50 strain was cultured in LB Broth at 38°C. Cultures were grown in liquid media at a culture density of 0.8 absorbance at optical density 600 [OD_{600}], exponential growth phase, before transferring to nematode growth media for *C. elegans* assays. Heat killed OP50 was heated in a water bath at 70°C for 30 min before being transferred to nematode growth media.

2.2.2.2 Nematode strains.

C. elegans strains were maintained with standard nematode culture techniques (Brenner, 1974) at 20°C unless otherwise stated. The wild type reference strain was (Bristol) N2. The *zdIs4* strain was from the *C. elegans* Genetics Centre (CGC) and contained an integrated plasmid containing an 1.1kb genomic fragment of the upstream promoter region of *hsp-4* fused to GFP (*hsp-4::GFP*). zdIs4 was expressed GFP at low levels across the worm; predominantly in cells adjacent to the gut. Upon increase in unfolded protein stress the cells increased GFP expression. The *zdIs4* strain was out crossed five times. The *gk514* strain from the CGC and contains a mutation in the *hsp-4* gene on chromosome II. The *gk514* strain was out crossed once. The *pmyo2::GFP* strain expresses GFP in the pharynx and contains a neuronal specific, partial rescue of *slo-1*.

2.2.2.3 Worm preparation.

All worms were transferred to separate culture plates 24 hours prior to experiments. Transferred worms were selected at larval stage 4 (L4) and allowed to mature into adult worms overnight. These adult worms were then used for the worm race, pumping and dispersal assays.

2.2.2.4 Ethanol concentrations in plates.

Samples of agar, 1cm x 1cm cubes, were collected from ethanol treated and untreated (control) test plates. Agar samples weights were recorded. Samples were sonicated for 1 h in 1 ml of ddH_2O. Sigma NAD-ADH test vials were made up to 16 ml with ethanol test buffer solution (100ml of 3.75g of glycine, 5.84g of NaCL in ddH_2O, made to pH 9 with NaOH). 20 µl of post-sonication sample solutions were added to 600 µl of NAD-ADH test solution. Samples of ethanol solution at concentration 0, 10, 20, 40, 100, 200 mM were used for calibration. 20 µl of the calibration samples were added to 600 µl of NAD-ADH test solution. All samples were replicated 3 times. All samples were then incubated at 37°C for 10 min before recording the

absorbance of each sample at 340 nm. Sonication of the agar samples removes the alcohol from the agar to the ddH_2O. The alcohol can then be treated with alcohol dehydrogenase (NAD-ADH test solution) for a set time and the absorption spectra of the reaction product measured. The absorbance gives an indirect measure of the alcohol present in the solution and therefore in the mass of the agar sample. Comparison against a concentration curve of known samples gives an indication of the concentration in the test plates.

2.2.3 Worm race assay

2.2.3.1 Worm race assay.

9cm culture plates were hand poured with 25 ml of NGM agar and left to set for 24h. 9cm plates were marked at two points at a distance of 5cm apart. The two points marked the *E.coli* food spot and the worm starting position. One point was seeded with 50 µl of *E. coli* OP50 at 0.8A [OD_{600}], ensuring that the *E. coli* was limited to a single culture spot. 6cm machine poured stock plates were seeded with a centrally placed spot of 50 µl of *E. coli* OP50 at 0.8A [OD_{600}] and incubated for 24 hrs.

Seeded plates were treated with ethanol to give a final concentration of either ~50 mM or 300 mM. Ethanol was pipetted directly onto the agar surface; volumes of ethanol were often such that the ethanol would cover the entire plate surface. Plates were incubated 24h for ethanol to equilibrate across agar, with plate lid covers sealed up until experimentation to avoid evaporation.

50L4 + 1 day worms were transferred to conditioning plates for 6 hours. Worms were rinsed off 6cm plates with 1ml of M9 and transferred into an Eppendorf tube. Worms were allowed to settle for 5 min and form a loose pellet before removal of the supernatant. A further 1ml of M9 buffer was added and left for 5 min. Supernatant was removed leaving a loose pellet of worms in approximately 30 µl of solution.

Worms were transferred to 9cm plates on the marked point 5cm opposite the food and the transfer spot allowed to dry. When no liquid remained timing for that food race began. The mean transfer rate for worms, incorporating losses from washing and transfer, was 60% (i.e. 60/100 worms). *E. coli* food spot was checked every 10 min for 2 hours and the number of worms recorded. The worms on the food were removed after counting at 10 min intervals. The number of worms left on the plate after 2 hours that have not reached the food spot was also recorded. A representative plate from the 6cm conditioning plates and 9cm race plates were kept after the assay to measure ethanol concentration.

2.2.4 Pumping assay

2.2.4.1 Pumping assay preparation.

Growth media used in all assays contained 0.8A [OD_{600}] OP50 grown for 2 days. All assays except food lawn assays used a 50 µl spot of OP50. Food lawn assays used a 100 µl OP50 spot spread over the entire culture plate with a glass plate spreader before 2 days growth. Ethanol was added 1 day before starting all assays. All assays were done under a temperature controlled environment at 21°C. Treatment types were blinded unless noted.

2.2.4.2 Pumping experiment.

Single adult worms were transferred to OP50 seeded plates for 5 min before recording pharyngeal pumping rate. Worms were transferred to an unseeded plate and allow to crawl free of bacteria for a further 5 min. Worms were then transferred to the unseeded test plates and pumping was recorded at 5, 20, 40 and 60 min time intervals. Worms were then transferred back to an OP50 seeded culture plate for a final 5 min before again recording pumping rate. Pumping rate was recorded for 1 min using a hand counter. Worm presence or absence from OP50 food spots at both beginning and end transfers was recorded where noted.

2.2.4.3 Pumping on food assay.

Single adult worms were transferred to a culture plate containing a lawn of OP50 bacterial food. After 5 min pumping rate was recorded using a hand counter for 1 min.

2.2.4.4 Pumping off food assay.

Single adult worms were transferred to an unseeded cleaning plate for 5 min to crawl free from bacterial food. Worms were then transferred to an unseeded culture plate for 60 min. Pumping rate was recorded at the 5, 20, 40 and 60 min intervals using a hand counter for 1 min.

2.2.5 Dispersal assay

2.2.5.1 Dispersal assay.

15 adult worms were transferred directly on the OP50 of a centrally seeded culture plate and the number of worms remaining on the food at 1 min or 5 min intervals, for the 10 min and 60 min durations respectively, was recorded.

2.2.5.2 Dispersal on heat killed OP50 food.

Worms were transferred to plates as in the dispersal assay but using heat killed OP50 and compared to experiments using live OP50.

2.2.5.3 Transferred food dispersal assay.

OP50 food was grown on separate culture plates, either pre-treated with ethanol or non-treated. Prior to running the dispersal assay the OP50 food spots were excised from the pre/non-treated plates and placed onto unseeded pre/non-treated destination plates. The OP50 was placed such

that the bacterial spots were pressed against the destination plate. Pressure was applied and given 1 min to settle before the OP50 was removed, leaving a transferred spot of OP50 on the destination plate. The dispersal assay was then run using the destination culture plates.

2.3 Results

2.3.1 Food race assays

C. elegans N2 strain were treated with varying concentrations of ethanol either during (intoxication), or for 6 hours prior (withdrawal) to, a food race assay. The food race assay quantifies the chemotaxic of the worm can be used in combination with ethanol to define distinct ethanol states. Untreated Worms had a mean 75% of the population reach the food after a period of 2 hours, N=16 (Figure 2.1A).

Worms treated with 50 mM ethanol during a food race after 6 hours conditioning with no treatment, an acute low dose of ethanol, were not significantly different from control worms receiving no treatment at 2 hours, [Unpaired t-test, $P>0.05$] (Figure 2.1B). After 2 hours a mean 72% of untreated, control worms made the food reward. Similarly after 2 hours a mean 72% of 50 mM ethanol treated worms made the food reward in the control paired race assays, N= 7.

Worms treated with 300 mM ethanol during a food race after 6 hours conditioning with no treatment, an acute high dose of ethanol, were significantly different from control worms receiving no treatment at 2 hours, [Unpaired t-test, $P<0.001$] (Figure 2.1C). After 2 hours a mean 68% of untreated, control worms made the food reward. In comparison after 2 hours a mean 3% of 300 mM ethanol treated worms made the food reward in the control paired race assays, N=6.

Worms treated with no ethanol during a food race after 6 hours conditioning with 300 mM ethanol, withdrawal from ethanol, were significantly different from control worms receiving no treatment at 2 hours, [Unpaired t-test, $P<0.05$] (Figure 2.2A). After 2 hours a mean 74% of untreated, control worms made the food reward. In comparison after 2 hours a mean 52% of 300 mM ethanol withdrawn worms made the food reward in the control paired race assays, N=10.

Worms treated with 50 mM ethanol during a food race after 6 hours conditioning with 300 mM ethanol, relief from withdrawal, were not significantly different from worms treated with no ethanol during a food race after 6 hours conditioning with 300mM ethanol, withdrawn, at 2 hours [1 way ANOVA with Bonferroni correction, $P>0.05$] (Figure 2.2B). Both relieved and withdrawn worms were significantly different from untreated, control worms at 2 hours [1 way ANOVA with Bonferroni correction, $P<0.05$]. After 2 hours a mean 77% of untreated, control worms made the food reward. In comparison after 2 hours a mean 56% of 300 mM ethanol withdrawn worms and a mean 60% of 50 mM ethanol relieved worms made the food reward in the control paired race assays, N=10.

Worms treated with 300 mM ethanol during a food race after 6 hours conditioning with 300 mM ethanol ("ethanol tolerant") were not significantly different from worms treated with 300 mM ethanol during a food race after 6 hours conditioning with no ethanol, an acute high dose of ethanol, at 2 hours [1 way ANOVA with Bonferroni correction, $P>0.05$] (Figure 2.3). Both tolerant and acutely intoxicated worms were significantly different from untreated, control worms at 2 hours [1 way ANOVA with Bonferroni correction, $P<0.001$]. After 2 hours a mean 79% of untreated, control worms made the food reward. In comparison after 2 hours a mean 5% of 300 mM ethanol tolerant worms and a mean 7% of 300 mM ethanol intoxicated worms made the food reward in the control paired race assays, $N=10$.

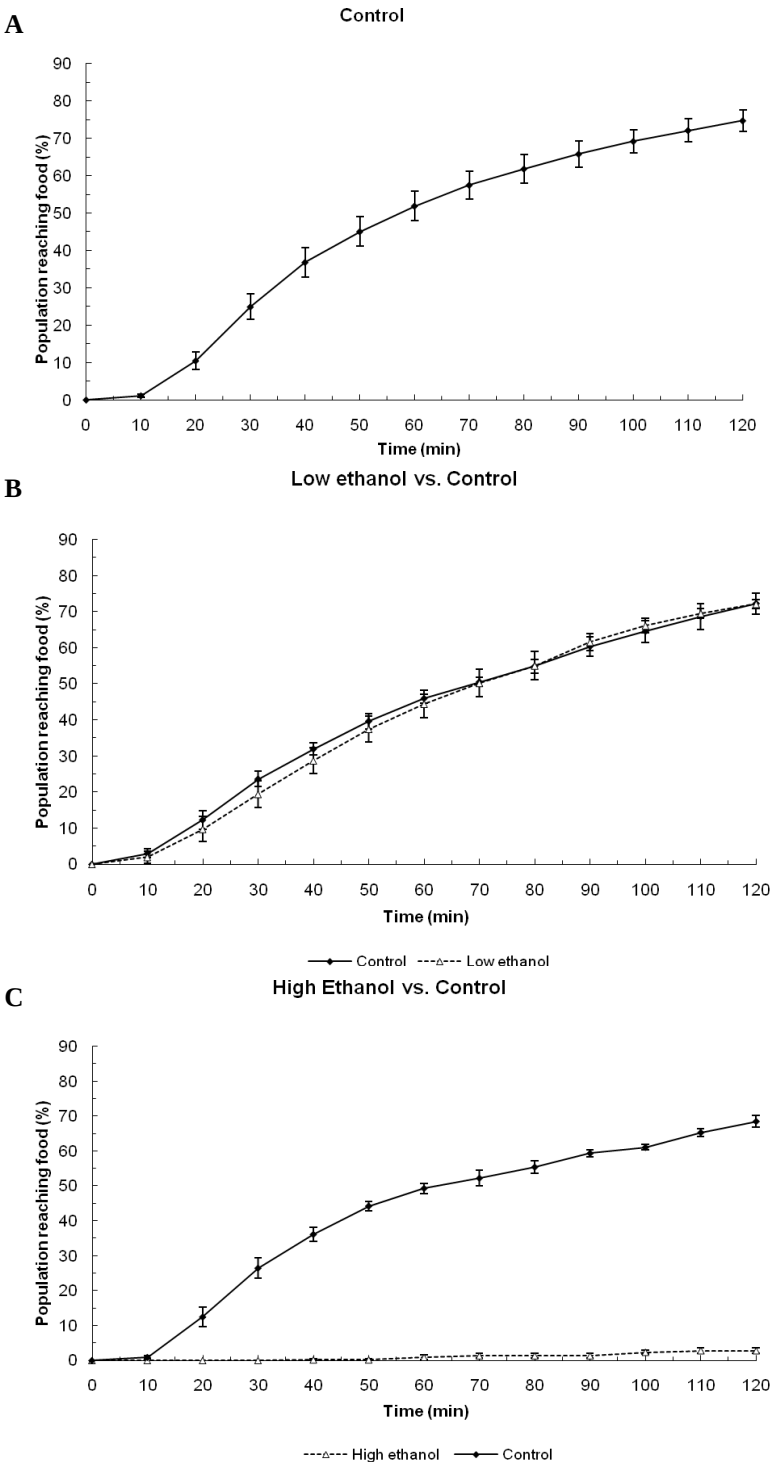
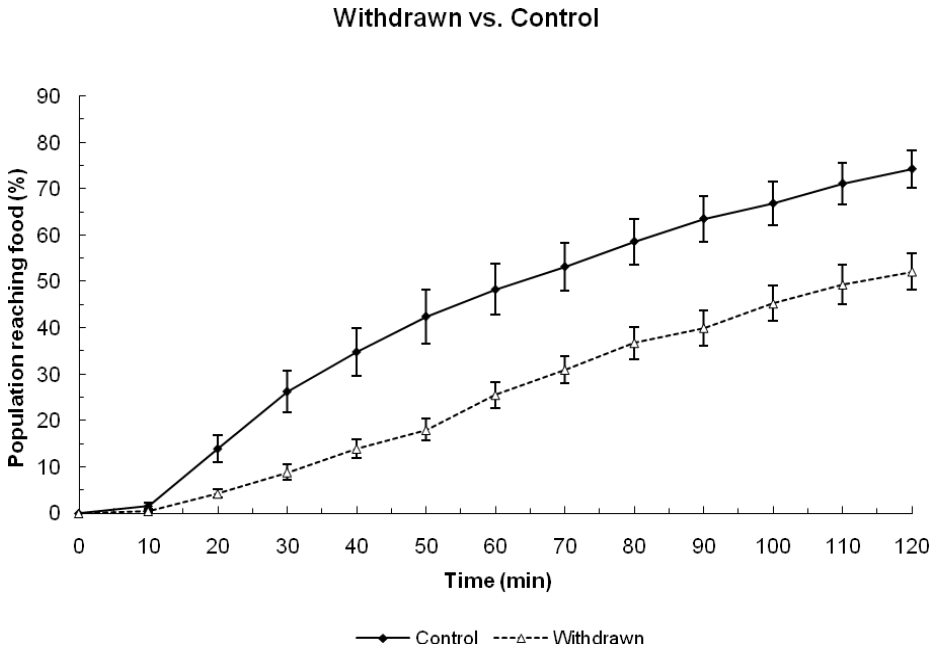


Figure 2.1. Food race of N2 worms on increasing ethanol concentrations.

N2 worms untreated were raced over a distance of 5cm towards a food reward. The population of worms reaching the food was recorded every 10 min for 2 hours, with the number of worms left after 2 hours also recorded. (A) No ethanol. 75% ± 2.2 SE of the population reached the food after 2 hours, N= 16; (B) N2 worms treated with 50 mM ethanol (Low ethanol) and no ethanol (Control). 72% ± 1.2 SE of 50 mM ethanol treated worms reached the food after 2 hours, whilst 72% ± 2.9 SE of untreated, control worms reached the food race after 2 hours, N=7. There is no significant difference between treatments; (C) N2 worms treated with 300 mM ethanol (High ethanol) and no ethanol (Control). 3% ± 0.9 SE of 300 mM ethanol treated worms reached the food after 2 hours, whilst 68% ± 1.7 SE of untreated, control worms reached the food race after 2 hours, N=6. Results are significantly different between treatments (Unpaired t-test, P<0.001.)

A



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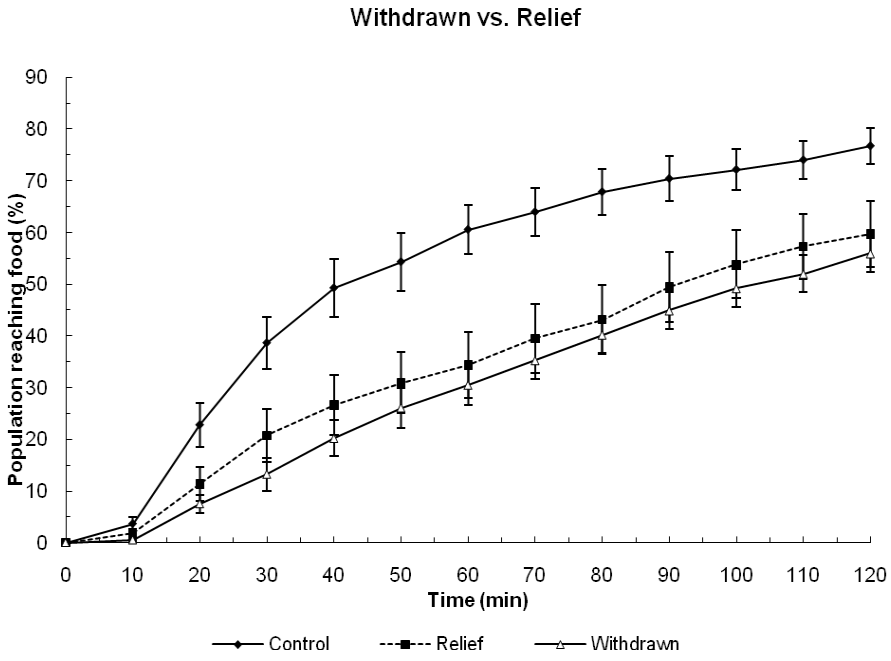


Figure 2.2. Effect of withdrawal from ethanol on food race assay.
N2 worms were raced over a distance of 5cm towards a food reward. The population of worms reaching the food was recorded every 10 min for 2 hours, with the number of worms left after 2 hours also recorded. (A) worms conditioned with 300 mM ethanol and then raced on no ethanol (Withdrawn), and untreated worms (Control). 52% ± 4.0 SE of 300 mM ethanol conditioned worms reached the food after 2 hours, whilst 74% ± 4.0 SE of untreated, control worms reached the food race after 2 hours, N=10. Results are significantly different between treatments (Unpaired t-test, P<0.05). (B) As in A, plus worms conditioned with 300 mM ethanol and then raced on 50 mM ethanol (Relief). 56% ± 3.6SE of 300 mM ethanol conditioned worms reached the food after 2 hours, whilst 60% ± 6.3 SE of 50 mM ethanol relieved worms reached the food. 77% ± 3.5 SE of untreated, control worms reached the food race after 2 hours, N=10. Results are not significantly different between ethanol treatments, but are significantly different from control worms (ANOVA, P<0.05).

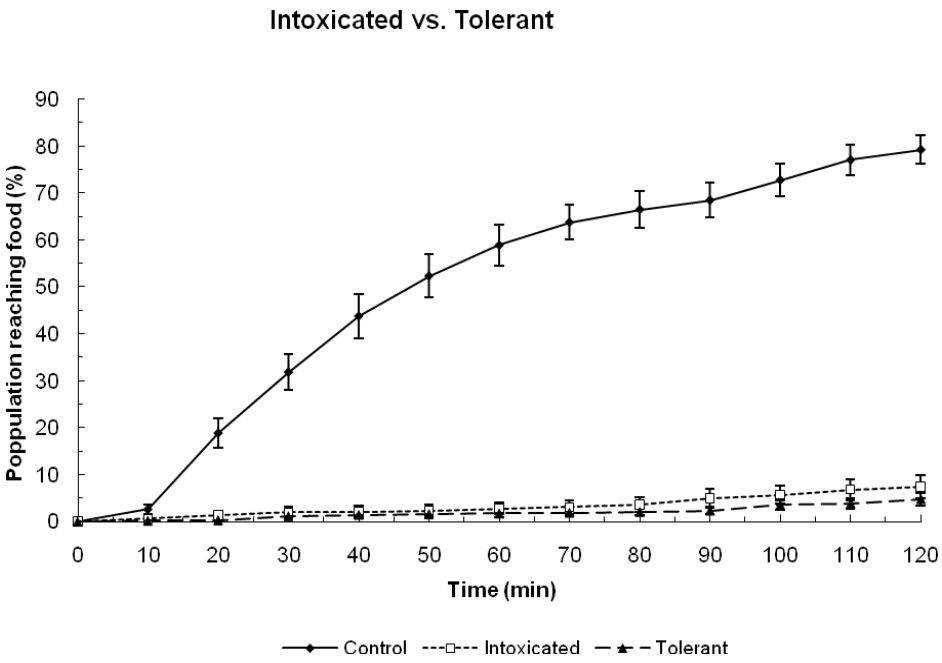


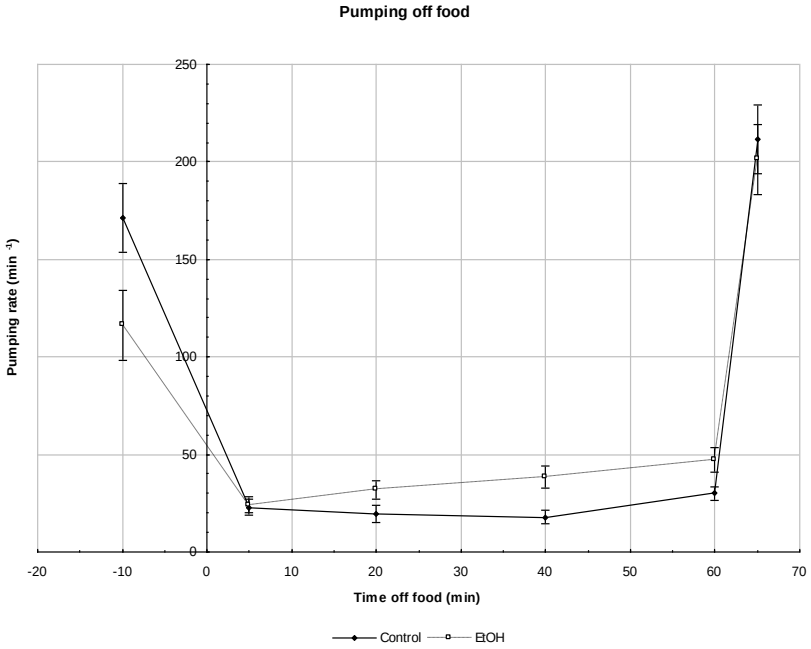
Figure 2.3. Effect of pre-conditioning with 300mM ethanol on food race assay.
N2 worms treated with 300 mM ethanol (Intoxicated), conditioned with 300 mM ethanol and then raced on 300 mM ethanol (Tolerant) and untreated worms (Control) were raced over a distance of 5cm towards a food reward. The population of worms reaching the food was reordered every 10 min for 2 hours, with the number of worms left after 2 hours also recorded. 5% ± 1.473 SE of 300 mM ethanol tolerant worms reached the food after 2 hours, whilst 7% ± 2.385 SE of 300 mM ethanol intoxicated worms reached the food. 79%± 2.983 SE of untreated, control worms reached the food race after 2 hours, N=10. Results are not significantly different between ethanol treatments, but are significantly different from control worms.

2.3.2 Investigating the use of pumping assays to define dose-dependent effects of ethanol

The initial experiments focused on the pumping rate of individual worms with and without food. Unpublished observations suggested that low dose effects <50 mM) could be resolved when using electrophysiological recording of pharyngeal pumping (James Dillon, pers. comm.). We tried to verify this using an assay featuring intact animals. Worms were placed on food for a 5 min period either side of a 1 hour period off food. The rate of pumping on and off food was observed over this period. Treatments included 50mM ethanol (Figure 2.4) and a higher 300mM dose (Figure 2.5).

There was an initial response to ethanol on food. Pumping rate is lower in 50mM ethanol treated worms (Figure 2.4) at the first time point. This response was lost at the later time point of 70 min when the worms are returned to food. Unrecorded observations during the non-blinded, trial phase (Figure 2.4A) showed that worms often moved from the food spot on which they were placed. It was hypothesized that the worms were showing a lower pumping rate because worms were slowing pumping in response to a non-food environment, not because the low concentration was inhibiting the pumping. This dispersal behaviour was investigated further.

A



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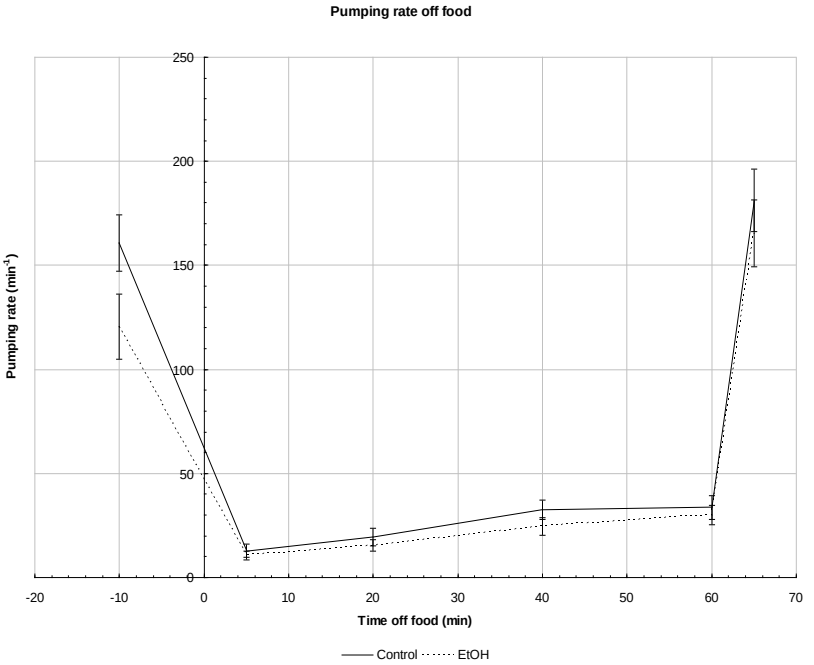


Figure 2.4. Pumping assay on and off food in the presence of 50mM ethanol.
A. Adult worms were treated with 50mM ethanol (Ethanol) or left untreated (Control). Pumping rate was measured on food after 5 min (-10 min), off food (5, 20, 40 & 60 min) and then back on food (70 min). Points represent mean pumping rate. Control N = 38, Ethanol N = 38. Error bars represent ± S.E.M. B. Repeat analysis, blinded to treatment type; return to food measurement taken at 65 min.

2.3.2.1 High dose effects

Treatment with high 300mM ethanol caused an inhibition of pumping both on, off and when returned to food (Figure 2.5). This is consistent with the previously reported dose-dependent inhibition of locomotion in food race assays (data not shown).

During the observations required to make pumping measurements on food, I noted that the worms dispersed from the food. The dispersal rates of the ethanol treated worms show a higher rate of dispersal than their control counterparts (Table 2.1). This confounds pumping measurements, as food is a major trigger for pumping.

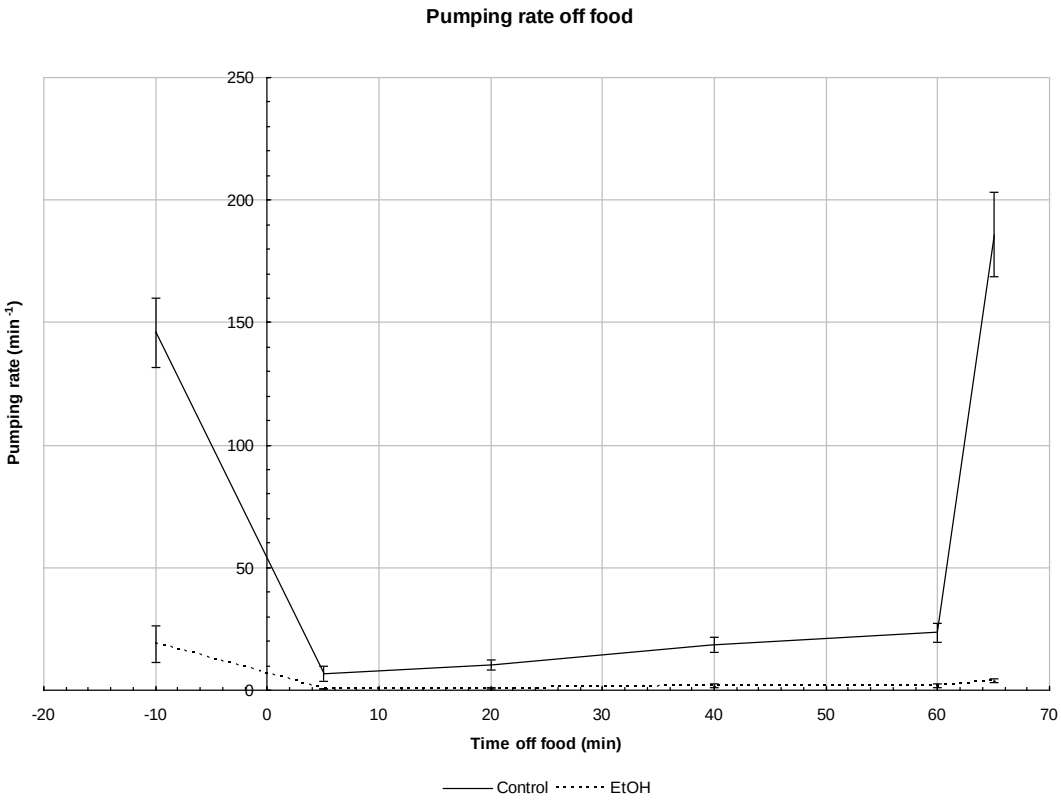


Figure 2.5. Pumping assay on and off food in the presence of 300mM ethanol.
Adult worms were treated with 300mM ethanol (EtOH) or left untreated (Control). Pumping rate was measured on food after 5 min (-10 min), off food (5, 20, 40 & 60 min) and then back on food (65 min). Points represent mean pumping rate. Control N = 38, Ethanol N = 38. Error bars represent ± S.E.M.

Table 2.1. Pumping assay dispersal from food, blinded.
Adult worms were treated with 300mM ethanol (EtOH) or left untreated (Control). Values represent dispersal over all repeats. Control N = 38, Ethanol N = 38.

Treatment	Worms off food (%)	
	-10 min	+65 min
Ethanol	78	56
Control	14	11

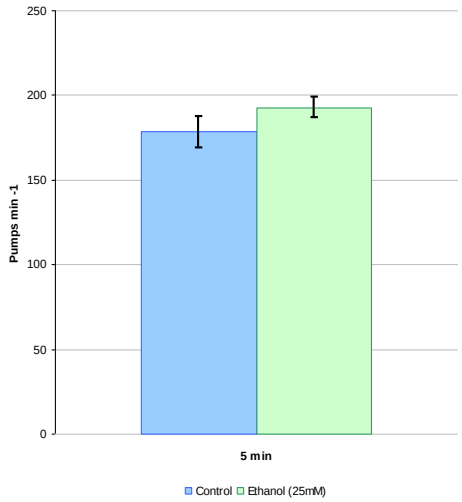
2.3.2.2 Pharyngeal pumping on bacterial lawns

To avoid problems with dispersal from food, pharyngeal pumping was also assayed on food plates seeded with a bacterial lawn. Worms cannot therefore 'escape' the food signal. Pumping was assayed at four ethanol treatment concentrations: 25mM, 50mM, 150mM and 300mM. Treatment groups were blinded. Of the 4 treatment groups none showed any significant difference from control treatments, apart from the 300mM ethanol treatment, which reduced

pharyngeal pumping (Figure 2.6). Median pumping in groups Control and 25mM ethanol were 209.5 and 202.5 pumps per minute; the distributions in the two groups did not differ significantly (Mann–Whitney $U = 1898$, $n_1 = n_2 = 60$, $P > 0.05$ two-tailed, P = 0.6101). Median pumping in groups Control and 50mM ethanol were 214 and 219 pumps per minute; the distributions in the two groups did not differ significantly (Mann–Whitney $U = 1982.5$, $n_1 = 50$, $n_2 = 68$, $P > 0.05$ two-tailed, P = 0.1236). Median pumping in groups Control and 150mM ethanol were 220 and 208 pumps per minute; the distributions in the two groups did not differ significantly (Mann–Whitney $U = 125$, $n_1 = n_2 = 16$, $P > 0.05$ two-tailed, P = 0.4641). Median pumping in groups Control and 300mM ethanol were 220 and 44.5 pumps per minute; the distributions in the two groups differed significantly (Mann–Whitney $U = 31.5$, $n_1 = n_2 = 16$, $P < 0.05$ two-tailed, P = 0.0003).

These results are in contrast to the previous experiments where worm dispersal behaviour led to a decreased pumping rate on food.

A



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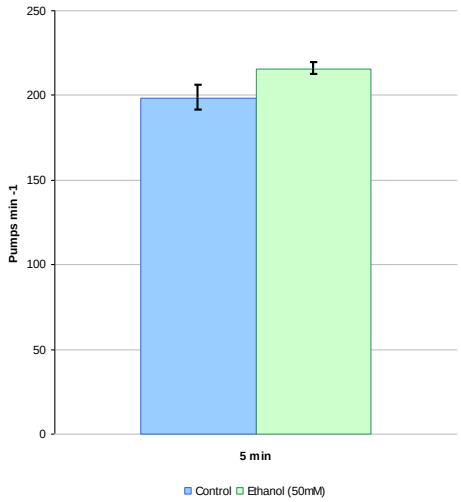

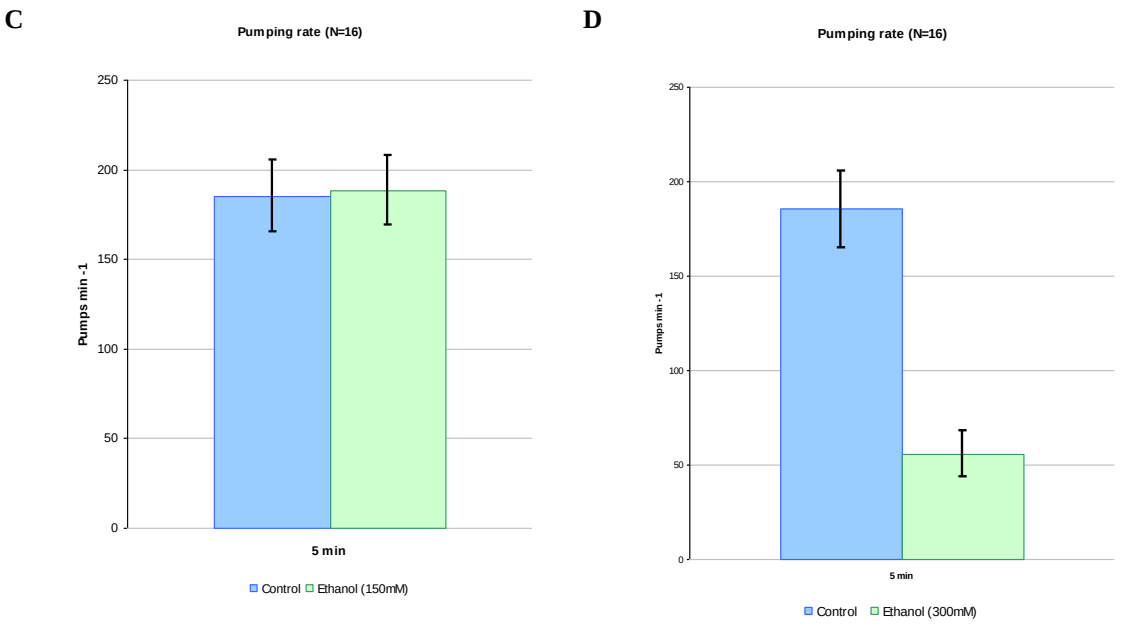

Figure 2.6. Effect of increasing ethanol concentrations on pumping rate on a bacterial lawn. Pumping assay on food, blinded. Adult worms were treated with 25mM (A), 50mM (B), 150mM (C) or 300mM (D) ethanol (Ethanol) or left untreated (Control). Pumping rate was measured on food after 5 min. Bars represent mean pumping rate. Control N = 60, Ethanol N = 60 (A), Control N = 60, Ethanol N = 68 (B), Control N = 16, Ethanol N = 16 (C) and Control N = 16, Ethanol N = 16 (D). Error bars represent ± S.E.M.

2.3.2.3 Pharyngeal pumping off food

Pumping off food at 150mM shows a slight increased pumping rate over control worms initially but soon decreases (Figure 2.7B). This may be a stimulatory effect of ethanol at a short time point before a more chronic inhibition of pumping later on. Adapted graphs from Figure 2.4 and Figure 2.5 are shown for comparison (Figure 2.7A & Figure 2.7C). It is worth noting that these graphs may not show any early time point stimulation of pumping rate due to an additional treatment in ethanol for 10 min prior, the food phase. The variability in the 150mM ethanol treatment is likely due to the lower N numbers.

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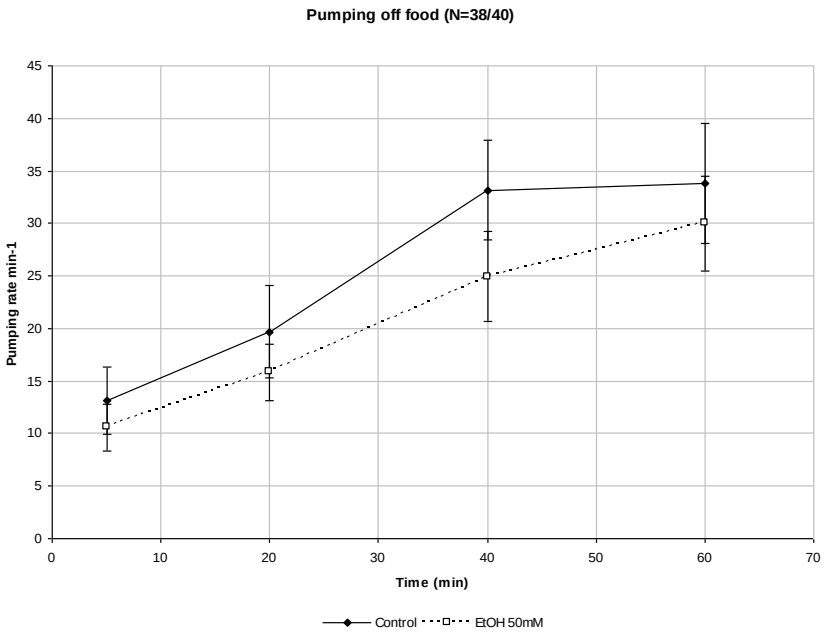

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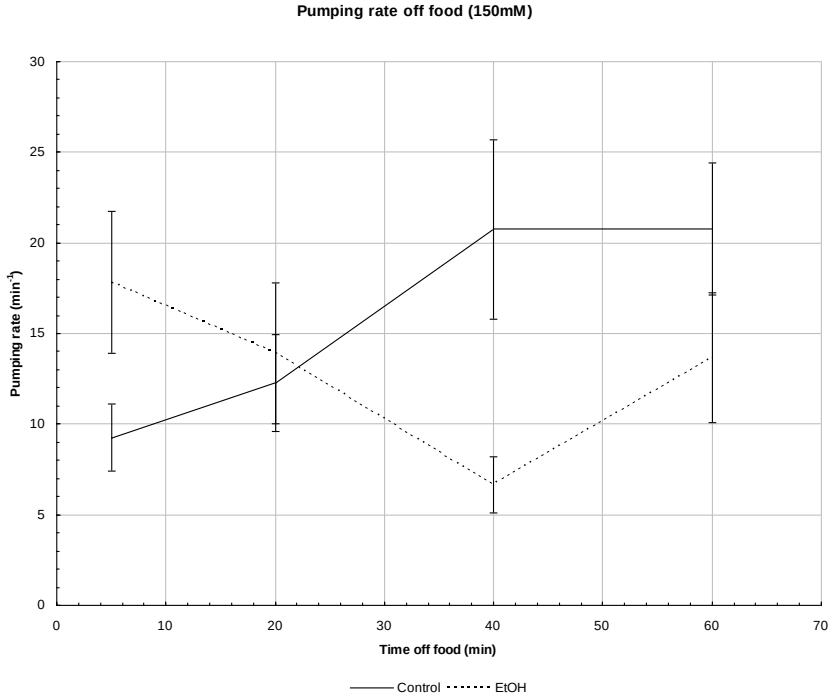

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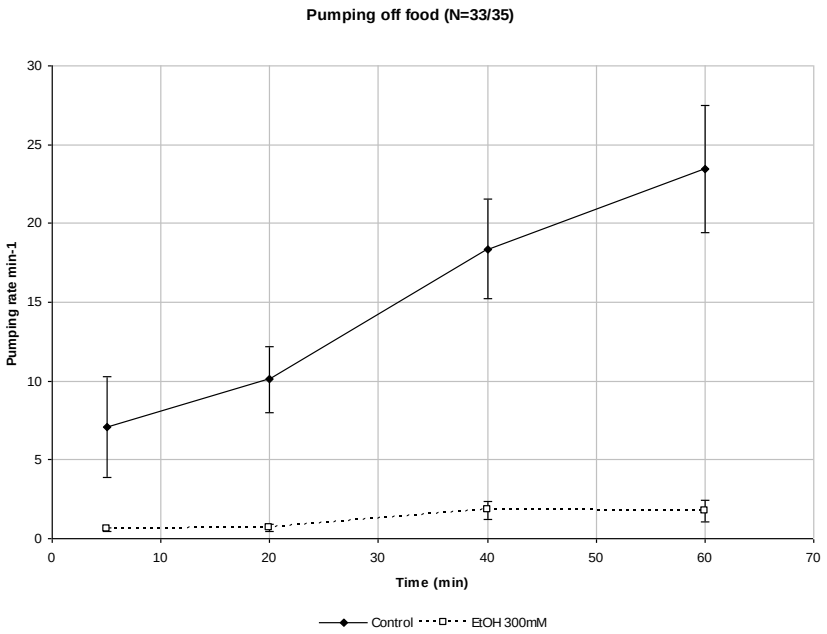


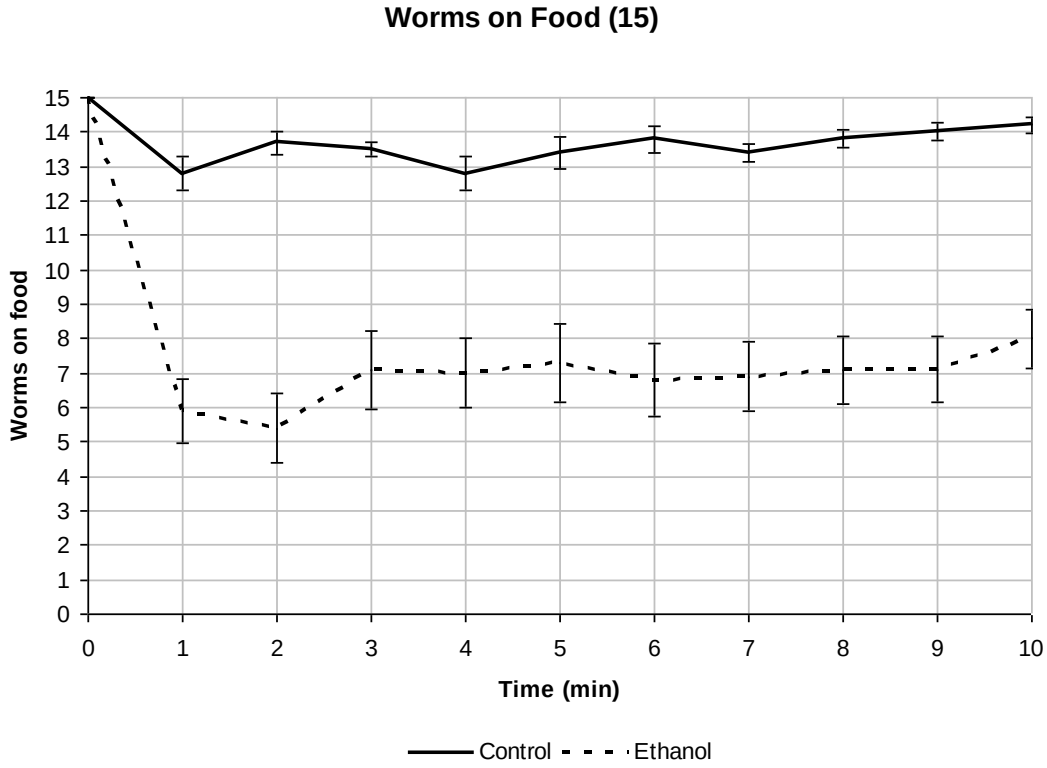
Figure 2.7. Pharyngeal pumping assay off food, blinded.
Pumping assay off food, blinded. Adult worms were treated with 50mM (A), 150mM (B) & 300mM (C) ethanol (EtOH) or left untreated (Control). 50mM (A) and 300mM (C) graphs adapted from Figure 2.4B and Figure 2.5 respectively. Pumping rate was measured off food at 5, 20, 40 & 60 min. Points represent mean pumping rate. Control N = 18, Ethanol N = 18 (B), Control N = 38, Ethanol N = 38 (A & C). Error bars represent ± S.E.M.

2.3.3 Worm dispersal: a novel assay for alcohol effects

In addition to informing the design of the pumping assays the dispersal offered a novel ethanol assay. We thus sought to define basis of the response. 15 worms were placed on a food spot, as in the pumping assay, and the number of worms that stayed on the food over time was counted. Worm dispersal was observed over a 10 min period on 50mM ethanol, and at a longer 60 min period (Figure 2.8). In addition the dispersal assay was extended to include a range of ethanol concentrations (0-300mM) over 10 min (Figure 2.9). Treatment type was blinded in all experiments.

Treatment with 50mM ethanol causes worms to move away from the OP50 food spot after just 1 minute. Approximately 50% of the population move away after this time and remain off for the duration of 10 min. Untreated worms have approximately 90% of the population stay on the food, with little variation over time. Extending the treatment duration from 10 to 60 min results in a slight increase in the worms that stay on the food, with around 75% of the ethanol population on returning to food after 60 min. Although individual worms were not recorded this suggests that they leave food and then move back towards it.

A



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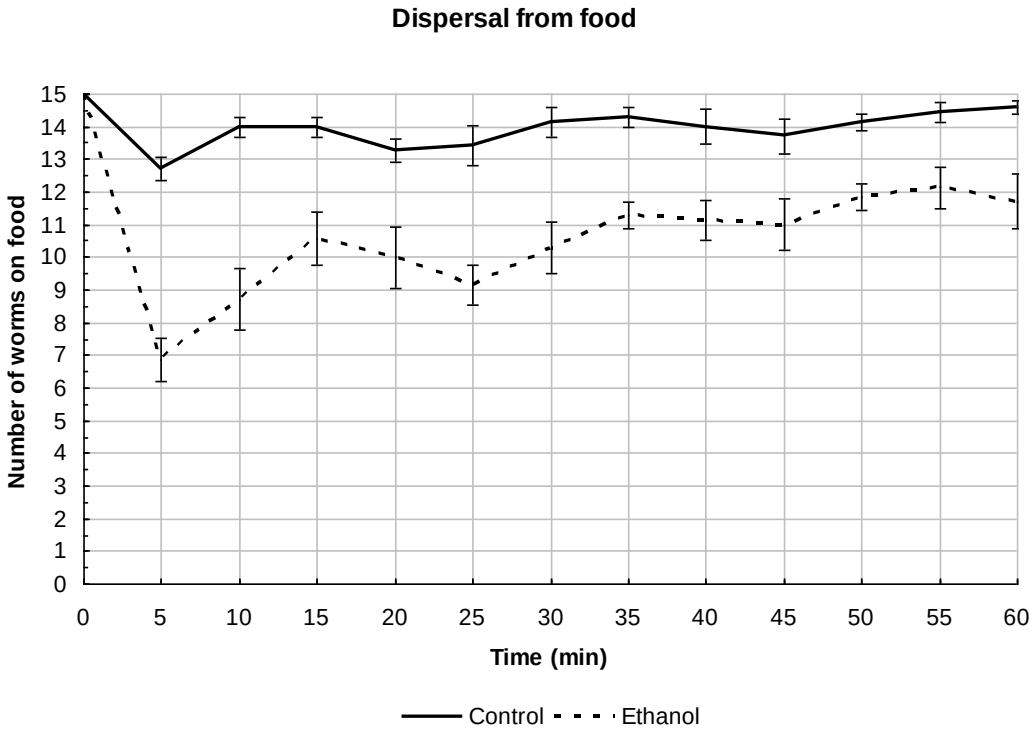


Figure 2.8. Dispersal from food assay for short and long time periods.
15 adult worms were treated with 50mM ethanol (Ethanol) or left untreated (Control) for 10 min (A) or 60 min (B). Dispersal rate from food was measured at 1 min (A) or 5 min (B) intervals. Points represent mean dispersal rate. Control N = 10, Ethanol N = 10. Error bars represent ± S.E.M.

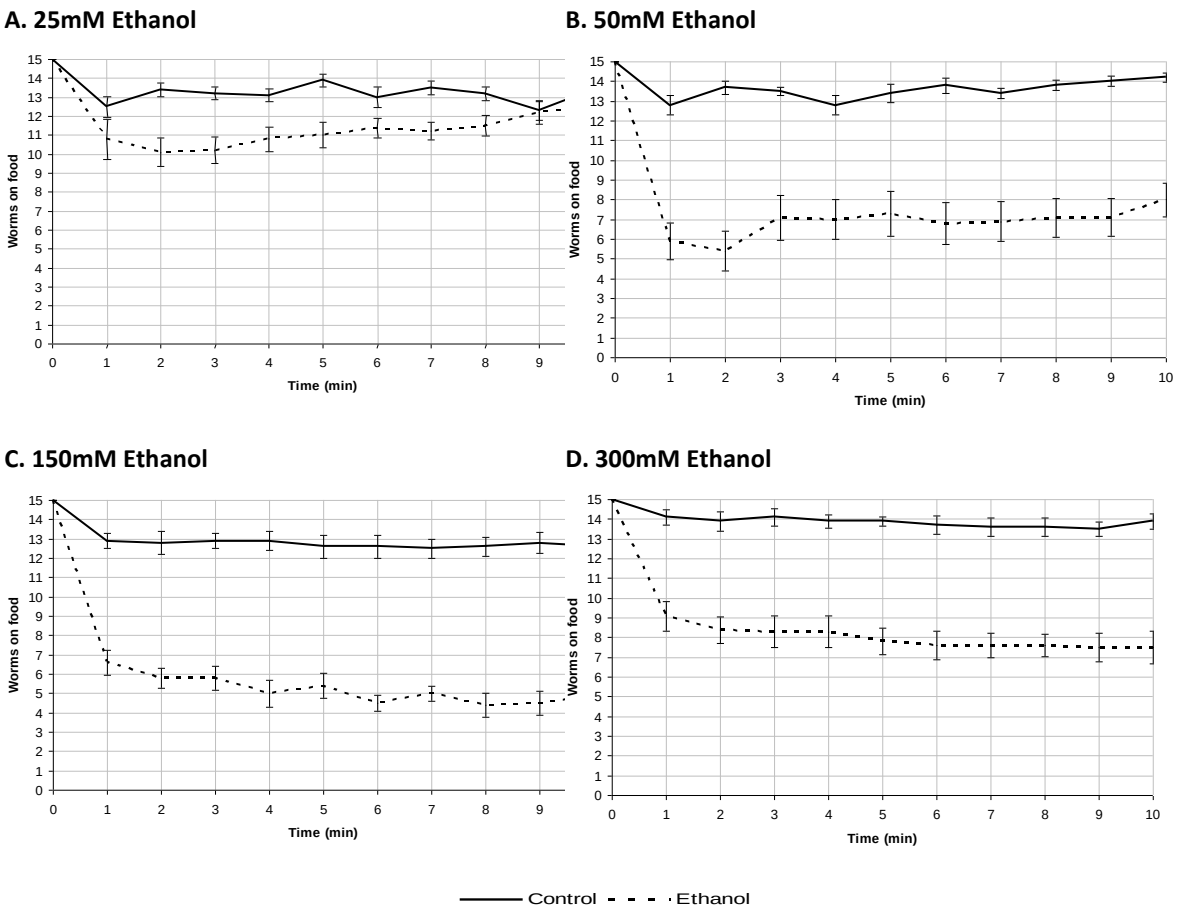


Figure 2.9. Dispersal from food assay for increasing ethanol concentrations.
15 adult worms were treated with 25mM (A), 50mM (B), 150mM (C) or 300mM (D) ethanol (Ethanol) or left untreated (Control) for 10 min. Dispersal rate from food was measured at 1 min intervals. Points represent mean dispersal rate. Control N = 10, Ethanol N = 10. Error bars represent ± S.E.M. 50mM graph (B) as Figure 2.8; repeated for comparison.

Increasing ethanol treatment causes *C. elegans* to disperse away from the food with a maximal dispersion at 150mM (Figure 2.9C). At 150mM approximately 35% of the worms are away from the OP50 food at 10 min. At the higher 300mM concentration the worms show a reduced propensity to disperse from the OP50 food, with perhaps ethanol inhibiting locomotion such as to hinder dispersal. The lowest ethanol concentration 25mM shows worms initially dispersing, but then recovering to baseline levels seen in control, untreated worms over time. This is likely the threshold for a dispersal phenotype.

There are 2 distinct possibilities which can explain the results above. Either the worm's behaviour is directly modified by ethanol interacting with the worm, or the presence of ethanol is changing the bacterial environment and indirectly modifying behaviour. To address this issue, an experiment was designed to investigate whether ethanol treatment had an effect on the OP50 bacterial food itself.

2.3.3.1 Worm dispersal on heat-killed bacteria

Worms were treated with either 50mM ethanol or no treatment in the presence of alive or heat killed bacterial food. If the bacteria were acting as a cue, rather than ethanol acting directly on the worms, then using heat killed bacteria, which cannot metabolise ethanol, would remove the dispersal phenotype. Likewise, if the worms were being affected by ethanol then heat-killed OP50 would not affect dispersal behaviour.

There is no observable difference between those on 50mM ethanol and dead OP50 versus dead OP50 without ethanol (Figure 2.10). Furthermore, worms fed heat-killed bacteria dispersed more that those fed live food, with or without ethanol. It is therefore it is not possible to discern whether or not they roamed away from ethanol in the presence of food because of an interaction between bacteria and ethanol generating an aversion substance. These results are consistent with the worms being neophobic, dispersing from novel environments. It could also be explained, however, by an in ability for the worms to recognise heat-killed OP50 as a food source.

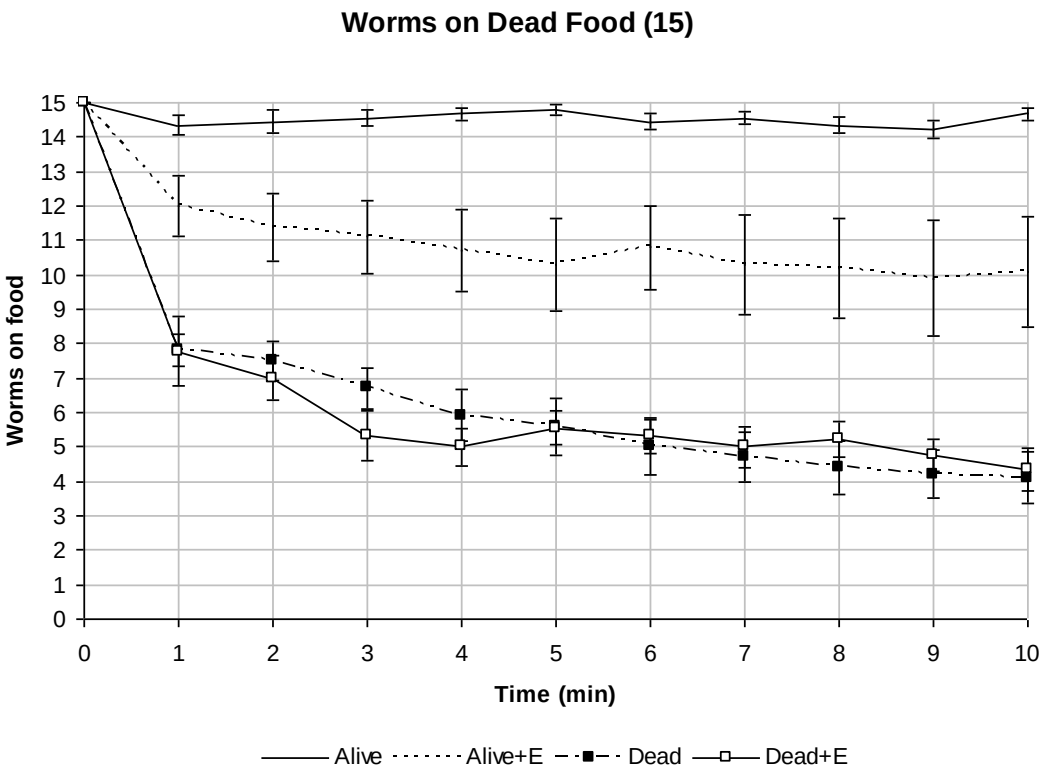


Figure 2.10. Dispersal assay with heat killed OP50.
15 adult worms were placed on live OP50 (Alive) or heat-killed OP50 (Dead) and on plates containing 50mM ethanol (+E) or left untreated for 10 min. Dispersal rate from food was measured at 1 min intervals. Points represent mean dispersal rate. Control N = 10, Ethanol N = 10. Error bars represent ± S.E.M.

2.3.3.2 Worm dispersal on OP50 incubated in ethanol

Separate to the experiment with heat killed OP50, bacterial food was either pre-treated with 50mM ethanol or not treated (Figure 2.11). The food was then transferred to a plate containing

or not containing 50mM ethanol. This was rationalized to allow food pre-treated with ethanol and transferred to a non-ethanol plate would cause worm dispersal if it was an indirect effect of the bacteria. Conversely if there was no dispersal then it would indicate no involvement of the bacteria, and may either be due to the environment or a direct affect.

Bacteria transferred after pre-treatment showed no differential effect on worm dispersal, whereas bacteria that was not pre-treated with ethanol but had ethanol present in the test plate showed worm dispersal consistent with previous paradigms. This result points to the bacteria not being responsible for any dispersal and indicates either an environmental or interaction driven response.

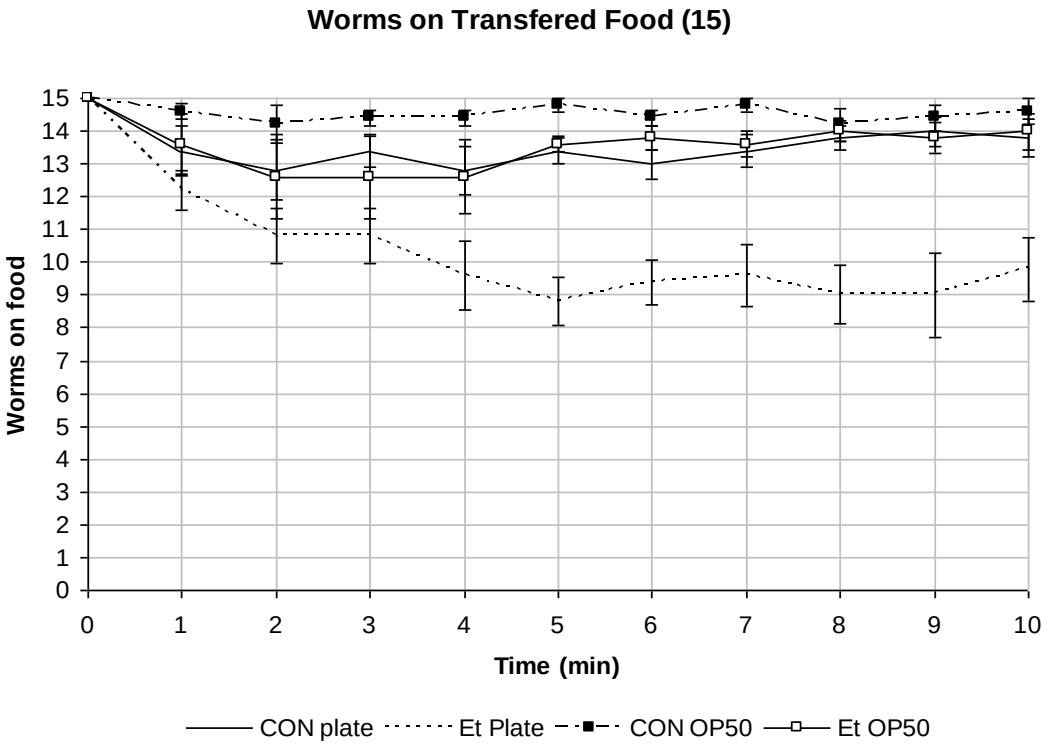


Figure 2.11. Dispersal assay with transferred OP50.
15 adult worms were placed on transferred OP50 either pre-treated with 50mM ethanol (Et OP50) or left untreated (CON OP50) for 10 min. 15 adult worms were also placed on transferred OP50 left untreated to plates that contain either 50mM ethanol (Et Plate) or no treatment (CON plate). Dispersal rate from food was measured at 1 min intervals. Points represent mean dispersal rate. Control N = 5, Ethanol N = 5. Error bars represent ± S.E.M.

The results indicate that there may be some behavioural response to ethanol intoxication, although this still needs to be separated from the environmental cue. The next step is to see if the response remains in the presence of a pre-condition treatment with ethanol to the worms.

2.4 Discussion

The results show that ethanol mediated behaviour can be modelled in the organism *C. elegans*. Treatment of ethanol at high concentrations shows a major depression in locomotion associated

with a reduced ability to reach a food reward after 2 hours. This affect is not seen at concentrations of ethanol less than 150mM. Removing worms from ethanol causes a withdrawn state which presents itself as a reduction in locomotion greater than that of control worms but not as great as ethanol intoxicated worms. The withdrawal response seen with longer conditioning times is that of a 'separate' response, and not of any lingering residual effect of alcohol still present in the worm. As withdrawal state worms show an improvement in race outcomes when reintroduced back to ethanol, it would not be expected in worms intoxicated with a reducing exposure to ethanol after removal. A withdrawn state can be partially rescued ("relief") in worms by treatment with a low concentration of ethanol, (Mitchell, unpublished data). Although not seen in these results, worms show an increased ability to make the food reward after 2 hours compared to withdrawn worms. It is likely that a conditioning time of 6 hours is not sufficient to produce a significant difference between withdrawn and relieved states. Increasing conditioning length to 12 or 24 hours should be able to distinguish responses. This may also be the case for worms that have been chronically exposed to ethanol to develop tolerance. No significance is seen between worms on chronic exposure to ethanol and those who have been acutely treated. By increasing the time conditioned in ethanol it should separate the responses so that tolerant worms are significantly different to acutely treated worms.

Looking at the pharyngeal pumping off and on food there seems to be a dose dependent inhibition with increasing ethanol concentrations reducing the pumping rate. This may be time dependent as early time points suggest a possible stimulatory affect. Looking at dispersal from food worms again show an increased rate of dispersal with increasing concentrations of ethanol. This does seem to be limited by the ability of ethanol to inhibit locomotion. It is still unclear as to why the worms disperse from food, although early indication point to a non-bacterial food dependent mechanism. It is interesting to note that heat-killed food also causes dispersal behaviour similar to ethanol treatment. It is likely that the environment is playing a role in food dispersal, with changing of food type or culture background causing an aversive response; neophobia. Both sets of experiments warrant further investigation.

Modelling the behavioural effects of ethanol in *C. elegans* allows an investigation into the genetic and molecular determinants underpinning these behaviours. By studying the effects of genetic and pharmacological manipulation on the different behavioural states, the molecular targets of ethanol can be discovered. This will be the experimental method used to look at other aspects of *C. elegans* ethanol biology in later chapters.

2.5 Future Work

Whilst the behaviours under low and high ethanol intoxication and on ethanol withdrawal are clear further work needs to be done to significantly identify the differences in relief and withdrawal and tolerance and intoxication. Increasing the ethanol conditioning time may

significantly separate these behaviours. Whilst these methods are becoming established we can use them to test and differentiate potential genetic targets for modulating the ethanol behavioural paradigms. Whilst the worm race paradigm offers a robust assay for understanding how ethanol works at acute and chronic treatments with relatively high dose ethanol concentrations, it is limited to using a population of worms. Future work should also focus on creating a sound paradigm dependent assay to use on individual worms.

It is important to define the dispersal response to ethanol as a function of behavioural change due to ethanol or environment. Pre-exposure to an ethanol source should clarify whether the response is a reaction to environmental change or a direct effect of ethanol on behaviour such as an increase in speed or inhibition of turns. Examining the effect of early exposure should also be a priority during food absence as it appears that there may be a slight increase in pumping rate during the first 10 min that is unaccounted for on 150mM ethanol treatment. Existing experiments failed to pick up this response as worms were being treated with ethanol prior to absence from food.

3 Bioinformatic Identification of Genes Involved In Ethanol Response

3.1 Introduction

The need to standardise data stems from the overwhelming variation in recording and analysing different variables. This problem is particularly pronounced in biology because of the complexity of the information and the ideas that are portrayed (Soldatova and King, 2005).By categorising information by certain defined criteria, it is possible to overcome the potential problem of analysing data from different sources (Thomas et al., 2007). The work done on how ethanol mediates its control over behaviour is vast in its scope and multifaceted in its evidence. By integrating this data into a database using consistent annotation practices, it is possible to analyse the whole rather than the sum of its parts. In this chapter, strictly defined information from articles on the molecular mechanisms of ethanol actions from across 5 species has been compiled in a controlled format for analysis. Combined with the Gene Ontology database information there is approximately 3500 records of evidence for over 900 biologically important molecules implicated in the action of ethanol within an organism. Information on treatment type, duration and ethanol concentration are also available for a number of records.

3.1.1 Ontologies

An ontology is a system of controlled vocabulary to allow organisation and retrieval of related information. The idea of an ontology is to define a specific vocabulary for information such that it cannot vary within a certain criteria, and is therefore not open to multiple interpretations. It also aids in the integration of new information and the search and retrieval of related information by automated processes (Rubin et al., 2008). The number of different ontologies in the public domain is increasing, which can make linking information more difficult. Not all ontologies that are created will follow the guidelines of others, including the use of controlled vocabularies. When two or more ontologies overlap in function, careful consideration needs to be made regarding the information to be mapped onto the ontology before selecting a particular ontological system in preference to another.

The use of an ontology ties in with the storage of information in databases. Often the ontology is used as a way of management and automated searching for a particular database. This is how the ontology system will help search and filter information from the ethanol database. Having an ontological based system also allows information to be easily searched or analysed in a high throughput manor (Beissbarth, 2006)

3.1.1.1 The Gene Ontology (GO)

The Gene Ontology (Ashburner et al., 2000) is the archetype biological ontology database, which maps the gene products of different species with information regarding three major components; cellular component, biological process and molecular function. A gene product is related to other gene products by its local within a cellular compartment, its process within that compartment and the molecular function of that gene product. These are mapped hierarchically for each gene in the form of parent-child relationships linked by identifiers to the relationship type. These are the following terms:

- is_a

- part_of

- develops_from

- regulates

- positively_regulates

- negatively_regulates

These relational identifiers allow information that is linked to be pulled out at any number of levels and to map down to the information that is related. This is a very powerful way of representing data and used by many ontologies.

In addition to being used to select candidate genes, GO has been integrated into the ethanol target database to permit queries using these pre-existing terms.

3.1.1.2 The Evidence Code Ontology (ECO)

Another important type of information that needs to be controlled is the type of change that is observed within the biological organism due to the ethanol exposure. This problem depends on the type of information disseminated, as different biological molecules vary in different ways as well as the way in which they were measured. A good start to defining the evidence is to say what is happening and how it was inferred. We use the Evidence Code Ontology (ECO) (Evidence Code, 2008) for the controlled vocabulary discussing how the evidence was inferred.

3.1.2 Molecular targets of ethanol

The information and data available on the responses to ethanol is overwhelming. Many different organisms, treatment methods and analysis have been used to characterise ethanol mediated behaviour and pathology. Handling the data with such a variety is a difficult process and is not made easier by the lack of an integrated resource for data mining. The aim of this investigation is to create a universal database which incorporates pre-existing information and allows further integration of new information such that data can be searched, queried and filtered for analysis. To ensure a set standard with information integration, data is stored according to ontological

design by which variation and redundancy is minimal. Several common pathways that respond to ethanol are conserved between species and are highlighted in section 1.4.

Because of the profound effect that ethanol has on the central nervous system a large chunk of the research in to the molecular mechanisms controlling behaviour have focused on classical neurotransmitter systems and intracellular signalling cascades. These make up the majority of the research articles that form the base of the databases dataset.

3.1.3 Homology mapping

Part of the motivation of this work is the translation of observations from one organism into other organisms for further analysis. Of particular interest here is the relationship between known ethanol targets in humans and model organisms, in particular *C. elegans*. By recording specific protein UniProt accession numbers, molecules from this database can be mapped on to homologues from other species. Of particular interest for this analysis are putative orthologues – gene/proteins that are related by a speciation event (i.e. shared a single common ancestral gene in the common ancestor of the two species being compared).

3.2 Methods

3.2.1 Source data

Data was gathered from 114 review articles dated from 2006 – 2009. Third party databases are queried for ethanol responsive proteins. Review articles obtained by keyword searches through the PubMed database. Articles are included based on the organism; *Homo sapiens, Rattus Norvegicus, Mus musculus, Drosophila Melanogaster* and *Caenorhabditis elegans*.

3.2.2 Database design.

The database was created using Microsoft Access 2003. Data is stored in nine Tables (Table 3.1) with a single main Input table storing the primary data (Figure 3.1). In addition to a unique entry identifier ("ID"), this table has fifteen input fields (Table 3.2). The Molecule table lists all the molecules (including UniProt Accession number where possible) that are currently associated with ethanol response, with the original name given in the study in the Input table.

3.2.2.1 Ontologies

Biological ontologies are used in this work to control the vocabulary of the input data as a way of data management, and to facilitate automated searching of terms. This database makes use of the Evidence Code Ontology (ECO) and Gene Ontology (GO) (Ashburner et al., 2000). ECO was used to control the terms used in the database, while GO was used to define some of the source data (see 3.2.1).

3.2.2.2 Molecules

To limit duplication and confusion, genes and proteins were converted to a "Common name", linked where possible to an unambiguous UniProt accession number. Chemicals were linked to the PubChem database accession numbers which are unique from the UniProt accession numbers.

3.2.3 Orthology mapping

Orthology mapping was performed by Dr Richard Edwards. Ensembl version 60 (accessed 2/12/10) was used to download predicted orthology relationships between the five main organisms in the database: Human, Mouse, Rat, *D. melanogaster* and *C. elegans*. PICR was used to map UniProt accession numbers from the ethanol database onto Ensembl gene identifiers. These links were used to map all possible proteins from the database on to human proteins for network analysis and visualisation.

3.2.4 Human network visualisation

Orthology-mapped human proteins were visualised as a set of hyperlinked web pages using HAPPI (http://www.southampton.ac.uk/~re1u06/software/happi/index.html) for further data exploration. These pages can be accessed at: http://www.southampton.ac.uk/~re1u06/research/bendb_html/.

3.3 Results

The original search for review articles generated a list of 113 articles. This dataset was narrowed down based on target organism, journal availability and language, to focus only on reviews focused on the specific action of ethanol in a molecular context between 2006 and 2009. For humans and rodents the articles were all review based whilst there were too few articles for flies and worms to do this, and thus the worm and fly data is from non-review articles. Articles on dual ethanol and burn treatment, cosmetics and ethanol manufacturing were excluded as non-relevant.

The database is an ongoing construction and the results presented here are best considered as a pilot study. In total, there are 3472 separate entries from across the five different organisms. There are 915 different proteins from these entries. These were gathered from 66 review articles and The Gene Ontology database query. The database includes the Evidence Code Ontology for standardising evidence input. An overview of the database design and relationships is given in Fig 3.1.

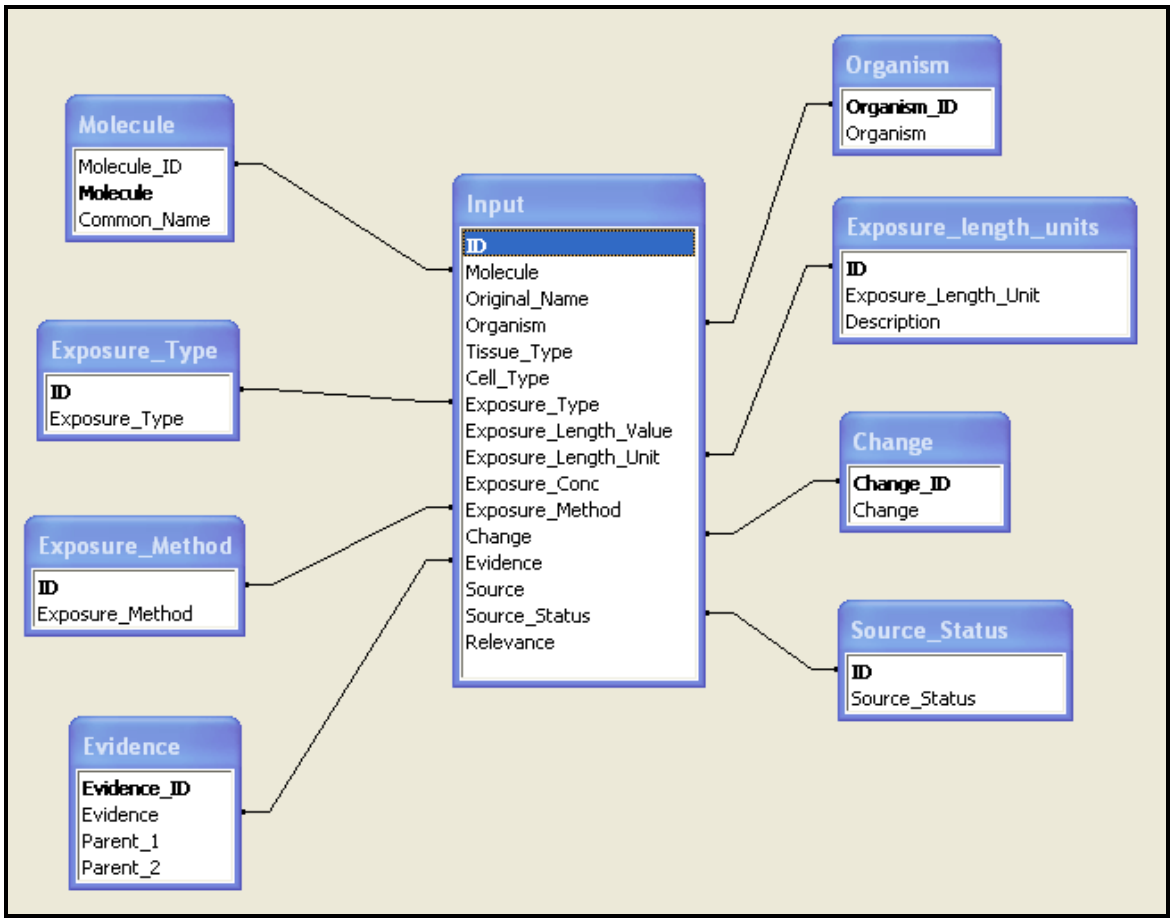


Figure 3.1. Main databases tables and relationships.
Descriptions of tables and fields are given in text, Table 3.1 and Table 3.2.

Table 3.1. Ethanol database tables.

Table	Description	Entries[1]
INPUT	Main entry table	3472
Organism	Experimental organism	6
Molecule	Molecule ID to common name and text description mapping	992
Exposure_Type	Exposure Type ID to text description mapping	5
Exposure_Method	Exposure Method ID to text description mapping	4
Exposure_Length_Unit	Description of distinct exposure units used	8
Evidence	OBO Evidence Ontology terms	148
Change	Phenotypic change ID to text description mapping	93
Source_Status	Source status ID to text description mapping	3

1. No. entries in table.

Table 3.2. Overview of fields in main database table.

Field	Description	Values[1]
Molecule	Protein, gene or other molecule affected	992
Original_Name	Molecule name given in source paper	902
Organism	Experimental organism included in the literature	5
Tissue_Type	Tissue implicated in the ethanol response as defined by the literature	110
Cell_Type	Cell type implicated in the ethanol response as defined by the literature	45
Exposure_Type	Nature of ethanol exposure (chronic, acute etc.)	5
Exposure_Length_Value	The numerical value of the exposure time used for ethanol treatment	n/a
Exposure_Length_Unit	Units of length of ethanol exposure	7
Exposure_Conc	Concentration of ethanol used in the experiment	119
Exposure_Method	Nature of exposure method (in vitro, in vivo etc.)	4
Change	Observed phenotypic (biochemical or behavioural) change associated with ethanol exposure and the molecule input	93
Evidence	OBO evidence code ontology (ECO) term	53
Source	Source PubMed ID, or the organism specific database source ID for third party database gathered information	1525
Source_Status	Database curation field (read or unread literature source)	3
Relevance	Brief text input to describe inclusion of a particular source or third party database search	n/a

1. No. distinct values in database.

3.3.1 Data entry

The aim of the database was to include a comprehensive summary of each piece of evidence, taken from a third party perspective that, in addition to providing a useful overview, would allow easy querying for specific information by users.

3.3.1.1 Molecules

In order to maintain comprehension and uniformity the data was limited to several variables which were identified before and during data entry. As the dataset was selected with a preference for molecular evidence; genes and proteins were the primary focus. This later expanded to include other molecules and chemicals, either intrinsic biological chemicals or externally derived pharmacological agents. To limit duplication and confusion genes were converted to proteins and the common name linked to the UniProt accession number used to identify the molecule. The chemicals were linked to the PubChem database accession numbers which are unique from the UniProt accession numbers. This process is not without caveats, as genetic mutants are not always a direct effect on the protein and thus linkage between what was actually studied and information in the database may be misinterpreted. To overcome this information about the change and the type of evidence, such as an up-regulation in an experiment from transcript levels were also recorded so that such information is not lost.

There were also problems associated with multiple subunit proteins and complexes, which are referred to as single entities in the mined information, but are recorded as separate proteins in the UniProt accession database. This was overcome by either citing the alpha subunit in homomeric complexes or not recording an accession number in multimeric complexes. Indeed this problem extended into data interpretation whereby authors would refer to a single protein as a specific entity, when several isoforms were present with specific functions and local. This again was overcome by either inferring subunit specificity in the dataset with clarification or deduction from the article at hand or simply not recording the information because of ambiguity. In addition this information was aided with the original author nomenclature recorded in the database such as to reduce error, or to correct at a later date when presented with further information.

3.3.1.2 Source organisms, tissue and cell type

Input data was restricted to that for one of five pre-selected organisms. Information from other organisms were rejected and not input into the database. If the organism information was not clear, as was the case with some of the papers on rodent studies, then the information was also not incorporated.

The tissue type and cell type were recorded also. These were not a problem except in one case where there was a mixed rat/human cell line used. This was input but did not have an organism input into its respective table field.

3.3.1.3 Treatment details

The treatment type tried to identify which type of treatment paradigm the research evidence was investigating. It was restricted to acute, chronic withdrawal and undefined. It was recorded if the publication specified the interest area, and was not assumed from the treatment duration. This was to avoid confusion between standardising between publications. Any assumptions that can be made on the does and time can be made from the raw data, where available, as to which paradigm in which organism was used. This is analogous to the recording of the proteins common name whilst still recording a unique protein accession number.

The value and duration of the treatment course were recorded in separate fields as to keep numerical and text data separate. Values were overlapping, e.g. 24 hours and 1 day, as to keep data entry quick, as conversions between values would have been lengthy. This process can still be applied later, which also makes examining individual records simpler at a later date. Treatment duration always focuses on the length of ethanol exposure. This is true for withdrawal treatment paradigms, but is complicated by the duration from ethanol before reintroduction in complex behavioural paradigms. Duration of abstinence is not recorded. For *in utero* based experiments assessing the risk of prenatal exposure to ethanol, the duration of alcohol was standardised to 21 days, the average period of gestation for the rodents. For human

in utero studies, no time data was included. Where ethanol concentration was given in the paper, it was recorded as described. Values were not standardised, and because of the complex nature of the values the data was stored as a text field. Some of the values were given as a concentration range, to avoid unnecessary data duplication.

3.3.1.4 Types of experiment

The method is a brief indication of the type of experiment done. The majority were *in vivo* experiments directly on an organism. Cell culture experiments were recorded as *in vitro*, although strictly speaking they are *ex vivo*. *In utero* describes the experiments done on ethanol exposure to pre-natal organisms. *In silico* was used to annotate which data was pulled from online databases. The experiments themselves may not be computer generated.

3.3.1.5 Ethanol effects

The change is a large set of different outcomes as a result of scientific scrutiny of a particular molecule. The list of changes was decided on from the information provided by data entry and was a rolling and adapting process of change type entry. Initial focus was placed purely on the proteomic level of change, with the hope of simply including up and down regulated molecules. This was quickly replaced by a need to include change not direct attributable to a regulation state of a single molecule, and started to incorporate more complex changes and phenotypes. The final adaptation was to include behavioural phenotypes of particular organisms under controlled situations. This has complicated the implications of how this data is analysed.

3.3.1.6 Evidence Code Ontology

The evidence field is based on the ontological system devised by the evidence code ontology. It lists a large number of evidence types in a hierarchical system to encompass the majority of molecular biology. It is this system that has been used to populate the evidence field. Not all evidence types were covered in the ontology, those that were not available as options were either excluded or labelled as a more baseline identifier.

3.3.1.7 Data sources

The source of the evidence was included as a PubMed ID pointing to the reference article. The data obtained from the GO database was kept in its original form of a unique organism database reference ID. Some of these were multiple articles separated by a pipe, they were not duplicated. The paper was labelled as read or unread to track changes in data entry. A final memo line was included to give an indication of why the original source was included in the dataset, again in order to track changes in data entry and database management.

3.3.2 Additional visualisation

As a resource for further investigation, orthology-mapped human proteins were visualised as a set of hyperlinked web pages using HAPPI (http://bioware.soton.ac.uk/software/happi/) for

further data exploration. These pages can be accessed at: http://bioware.soton.ac.uk/research/bendb/.

3.4 Discussion

The current work represents a pilot study, with further work needed to complete information entry into the database. Integration of information in to the database was the slowest, rate determining step. Manual data mining from the reference articles was an arduous task complicated by the variation in information presentation and nomenclature. Information gathering and input is a time- and labour-intensive task, which may benefit from a more community-driven "wiki" approach in future. Additional developments in the realm of text mining might also be of benefit to this project; the current setup can incorporate data with different degrees of confidence, which might enable to the visualisation of larger trends. Such data sources might also help overcome the inevitable ascertainment bias that arises when relying on review articles that have invariably been prepared with certain conscious or subconscious biases.

The current database structure is functional but could benefit from the integration of further biological ontologies; these should allow better visualisation of information output and advanced query function. As information was added to the database, the design itself had to be updated. Although undesirable, and potentially creating some inconsistency in the data stored, such changes are inevitable in this kind of project. It also became apparent that the degree of detail hoped for in terms of both ethanol treatments and effects was often absent, particularly from review papers. Given the difficulty in measure precise concentrations or durations for certain experiments, the inherent ambiguities and sometimes subjective reporting associated with behavioural responses, and the difficulty in conclusively linking responses to specific molecules, these limitations were to be expected but the scale of them was still a little disappointing.

3.5 Future Work

To fulfil the initial goals of the database, data from additional papers needs to be added to the database. Once all the information has been input then effort can be concentrated on data output and visualisation. The database requires the use of several ontological structures to manage information. One goal for future work is to integrate the ontologies into the structure of the database so that information can be queried and filtered through the ontological hierarchies. Another aim is to create a database front end so that information can be retrieved by users or so that information can be both retrieved and integrated by users.

4 The Unfolded Protein Response and Ethanol

4.1 Introduction

4.1.1 Ethanol as an agent of stress

Stress is an important component of the psychological and molecular response to ethanol. Ethanol intoxication, particularly at higher doses, engages regimes that are recognized as cellular stresses. Classic among these are the heat shock systems in which cellular stress of heat shock induces several molecular pathways including HSP-90, 70 60 and 40 and a number of small heat shock proteins. These routes are often engage additionally as response to cellular stressor including oxidative stress protein misfolding and toxicological activation. This is recognized by several studies that identify robust stress responses upon ethanol intoxication. Less is known if the reactive responses that underlie withdrawal and tolerance engage such pathway. Nonetheless, the induction of these pathways during the intoxication regimes that lead to withdrawal and tolerance makes this formally possible. Indeed, the Heat shock responses are often associated with protective pathways that operate during preconditioning protection. These stress pathways are known to modulate cellular stresses of the nervous system and potentially underpin cells and pathways implicated in ethanol effects. One such pathway that is poorly understood with respect to ethanol is the Unfolded Protein Response (UPR).

4.1.2 The Unfolded Protein Response (UPR)

The UPR is an evolutionary conserved mechanism for the homeostatic response to an increase in perturbed secretory and transmembrane proteins. Increases in protein load, dysregulation of calcium levels, altered endoplasmic reticulum redox environment, malfunctioning protein degradation and immune insult all trigger the UPR. An array of chaperone proteins, proteins linked to the secretory pathway and scavenging proteins all work to normalise stress condition within the endoplasmic reticulum. If ER stress remains unaltered then programmed cell death is triggered. Chronic ethanol conditioning has been shown to induce an UPR in several organisms including *Caenorhabditis elegans*. It is unclear, however, whether ethanol triggers an unfolded protein response at concentrations that cause behavioural responses to ethanol, and whether the UPR modulates these behavioural phenotypes.

The UPR was first recognised as an adaptive response to nutrient deprivation and misfolded protein load in the organism *Saccharomyces cerevisiae* (Kozutsumi et al., 1988). Identification of the processes triggering the UPR, the proteins involved in regulating transcriptional events and the identification of a consensus regulatory sequence upstream of target genes allowed for the characterisation of this separate response (Cox et al., 1993; Cox and Walter, 1996; Dever et al., 1992; Yoshida et al., 1998). Parallel work identified and characterised the *S. cerevisiae*

pathway in mammalian cells and demonstrated the conservation and importance of the UPR (Harding et al., 1999; Haze et al., 1999; Wang et al., 1998).

Typically the UPR response is triggered by an increase in misfolded protein load within the ER (Kohno et al., 1993); sequestering chaperone proteins and straining the protein degradation process. An increase in misfolded proteins can be caused by a perturbed folding system, glycosylation system or ER homeostasis mechanisms which act to keep the endoplasmic environment as favourable to protein folding as possible; such examples include calcium levels (Bonilla et al., 2002; Hojmann Larsen et al., 2001) and redox conditions (Trotter and Grant, 2002). Aggravation of endoplasmic reticulum associated degradation (ERAD), the process of removal and destruction of misfolded proteins, also triggers the UPR (Mouysset et al., 2006; Sasagawa et al., 2007; Tcherpakov et al., 2008). Other triggers of the UPR include infection with pathogens (Bischof et al., 2008; Haskins et al., 2008), nutrient deprivation (Fernandez et al., 2002a; Kozutsumi et al., 1988), heavy metal toxicity (Liu et al., 2006; Yokouchi et al., 2007) and perturbed ER protein trafficking (Higashio and Kohno, 2002; Sato et al., 2002).

In addition to moderating environmental responses components of the UPR are constitutively activated at basal levels to help control protein synthesis, development and cell differentiation (Sharkey et al., 2008; Sugiura et al., 2009). Failure of the UPR to maintain normal levels of misfolded proteins activates apoptosis (Hetz et al., 2006). The UPR is also seen as an important survival switch for tumourgenesis, allowing the extended life of cancer cells beyond detrimental conditions.

The unfolded protein response has also been implicated in the physiological pathogenesis of several diseases including chronic alcohol consumption, playing an important role in the development of liver damage, possible neurodegeneration, glucose/insulin dysregulation and alcoholic cardiomyopathy. Work done in the model organism *C. elegans* has shown a link between the UPR and ethanol exposure (Kwon et al., 2004; Uccelletti et al., 2004). *C. elegans* presents a useful platform for studying the effects of ethanol on the UPR because of the genetic tractability of the organism and the wealth of work already acquired on both ethanol exposure and the unfolded protein response. This review outlines the current knowledge of the unfolded protein response, similarities in *C. elegans*, the link between ethanol and the UPR and the use of *C. elegans* to dissect the role of the UPR in ethanol consumption. In addition the link between ethanol and the unfolded protein response in *C. elegans* is investigated, exploring the role the UPR may have in mediating the behavioural responses to ethanol.

4.1.3 Overview of UPR activation in humans/mammals

Primary initiation of the UPR is through the increased binding of Binding immunoglobulin Protein (BiP) to unfolded proteins in the ER (Fig 4.1). Binding of BiP to unfolded proteins sequesters it away from its alternate residency on ER membrane bound proteins

Serine/threonine-protein kinase/endoribonuclease 1 (IRE1α), Cyclic AMP-dependent transcription factor 6 (ATF6α) and PRKR-like endoplasmic reticulum kinase (PERK) whose activation are repressed by bound BiP (Bertolotti et al., 2000; Sommer and Jarosch, 2002). These three transmembrane proteins represent the three main pathways of the UPR; although there is current work to highlight the less well understood areas of UPR activated pathways (Caruso et al., 2008).

Upon increase in ER misfolded proteins BiP dissociates away from membrane bound PERK, binding the misfolded proteins. Dissociation of BiP then allows for subsequent dimerisation, and trans-auto phosphorylation of the membrane bound PERK protein (Ma et al., 2002). Activation of PERK initiates a kinase function in which it phosphorylates serine residue 51 on eukaryotic translation initiation factor 2 subunit alpha (eIF2α) (Harding et al., 1999). Phosphorylation of eIF2α allows for an attenuation of general protein translation through disruption of the 43S translation-initiation complex (Kimball et al., 1998), and an initiation of selective translation of UPR regulated transcripts. This may be mediated through internal ribosomal entry sites (IRES) or upstream open reading frames (uORF) within the 5'-untranslated region of specific mRNAs (Fernandez et al., 2002b; Lee et al., 2009). In addition PERK can also phosphorylate the transcription factor Nrf2, disassociating Nrf2 away from a complex with Keap1, and activating transcription of proteins involved in redox homeostasis (Cullinan and Diehl, 2004; Cullinan et al., 2003).

The release of BiP from membrane bound IRE1α also allows for dimerisation and trans-auto phosphorylation, activating its cytosolic domain which has ribonuclease activity (Tirasophon et al., 2000). The ribonuclease activity of IRE1α allows it to cleave the mRNA transcript of xbox-binding protein (XBP1). IRE1α ribonuclease removes a 26 nucleotide intron from the mRNA; causing a frame shift in the XBP-1 transcript and a change in size from 261 to 376 amino acids (Yoshida et al., 2001). IRE1α cleavage of *xbp1* mRNA leads to selective translation by the aforementioned phosphorylated eIF2α. XBP1 is a potent transcription factor that binds the consensus sequence $CCAATN_9CCACG$, known as the ERSE sequence. ERSE is located upstream of many of the UPR regulated genes involved in protein folding, chaperone function and ER homeostasis (Yoshida et al., 1998). XBP1 may also bind the unfolded protein response element (UPRE) [TGACGTGG/A], which also lies upstream of UPR associated proteins (Wang et al., 2000; Yoshida et al., 2003). IRE1α may also use its ribonuclease activity to degrade specific mRNAs, relieving the strain on peptide folding and translation (Hollien and Weissman, 2006).

ATF6α release from the ER membrane is promoted by BiP dissociation. Upon release ATF6α relocates to the Golgi apparatus where it undergoes cleavage and processing by two membrane-bound proteases; S1P and S2P which remove an N-terminal 50kDa peptide sequentially. This cleavage allows p50ATF6α to bind the CCACG portions of the ERSE and ERSE II sequences

[ATTGG-N-CCACG] with the help of NF-Y binding the CCAAT region, promoting transcription of UPR associated genes (Kokame et al., 2001).

The promotion of UPR specific genes is associated with an increased need to allow for protein degradation through ERAD, protein secretion, amino acid synthesis and cell cycle associated proteins. A great number of proteins are differentially regulated and have been characterised in several organisms.

4.1.4 The UPR and disease

The unfolded protein response is a complex mechanism for maintaining cellular homeostasis and is prone to disruption and malfunction. A wide range of insults can trigger the UPR, making the likelihood of its involvement in disease pathology quite high. Some of these triggers and diseases are explored in more detail below.

4.1.4.1 Hyperhomocysteinemia

Hyperhomocysteinemia and lipid metabolism perturbations are both risk factors for atherosclerosis. Hyperhomocysteinemia can trigger the UPR (Outinen et al., 1999) and lead to problems in lipid regulation and cholesterol synthesis (Hamelet et al., 2007; Werstuck et al., 2001). Hyperhomocysteinemia also contributes towards liver steatosis, large vacuoles of lipids developing within hepatic tissue. Homocysteine is believed to regulate lipid synthesis and scavenging through the protein SREBP and the UPR (Kaplowitz and Ji, 2006).

Excess exogenous and endogenous sources of saturated fatty acids have been shown to trigger the UPR in *S. cerevisiae* (Pineau et al., 2009). There may be a link between the UPR and the pathology of diseases associated with a high fat diet, such as cardiovascular disease and diabetes. It has been shown that cholesterol overloading has the ability to induce the UPR, and that atherosclerotic plaque growth and macrophage apoptosis may be an impact of this (Devries-Seimon et al., 2005; Tabas et al., 2007). The apoptosis regulator CHOP is thought to play a positive role in late stage atherosclerosis plaque growth and necrosis (Thorp et al., 2009).

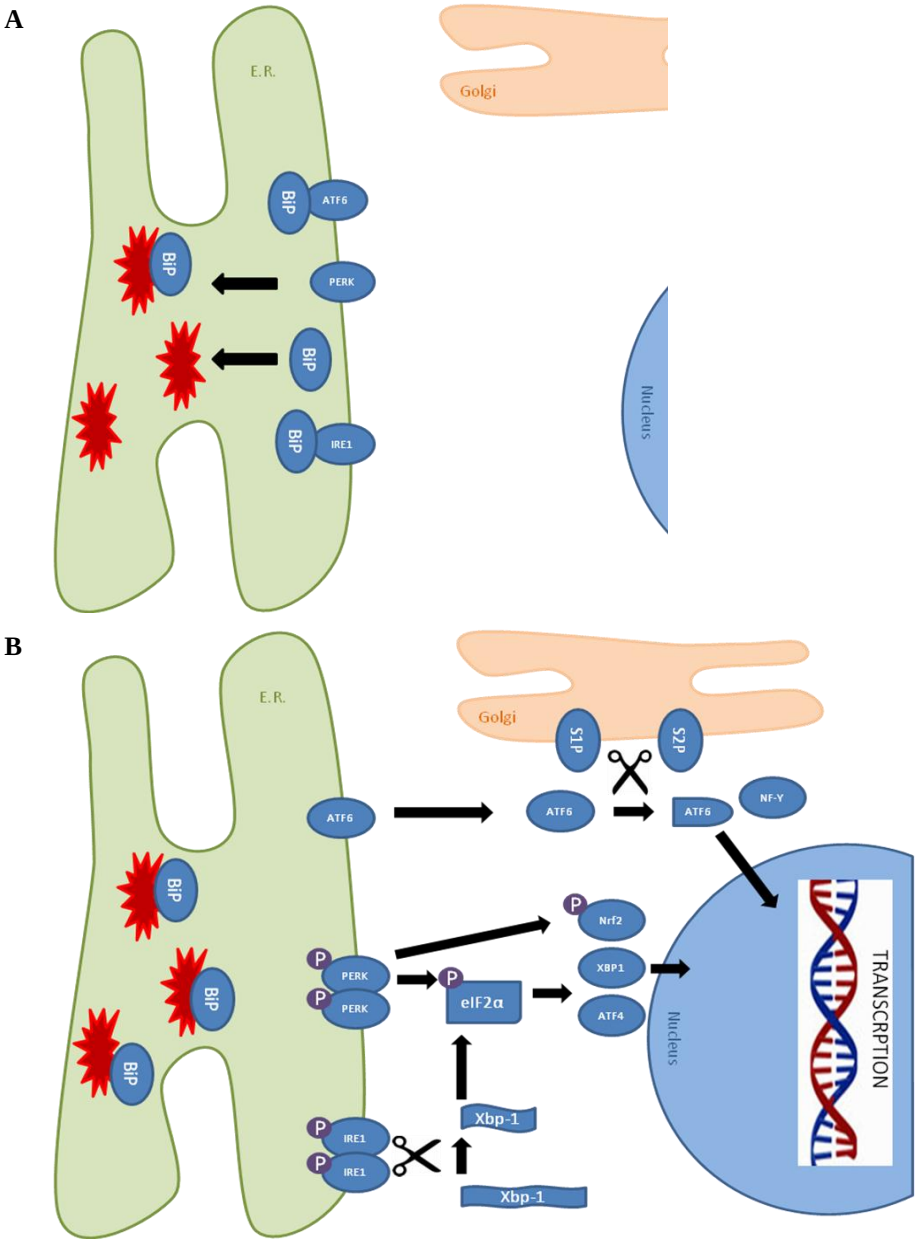


Figure 4.1. The unfolded protein response.
A. Repressed state of the UPR. Proximal sensors ATF6, PERK and IRE1 remain inactivated on the endoplasmic reticulum membrane whilst bound to the ER chaperone BiP. Increases in misfolded proteins, red shapes, promotes BiP disassociation from membrane proteins. B. Activation of the unfolded protein response. Increases in misfolded proteins cause the disassociation of BiP away from the proximal sensors on the ER membrane. IRE1 and PERK dimerise and auto-phosphorylate on activation. IRE1 can then cleave *xbp-1* mRNA. PERK phosphorylates the transcription factor Nrf2 and translation control protein eIF2α, purple circles. eIF2α attenuates general translation whilst activating translation of UPR specific transcripts such as ATF4 and XBP1. ATF6 translocates away from the ER and is cleaved by S1P and S2P proteases on the Golgi. ATF6 50kDa fragment then activates transcription with NF-Y. Transcription activates UPR specific genes controlling ER homeostasis and protein folding control, such as BiP.

4.1.4.2 Diabetes

Diabetes is caused by a lack of, or lack of response to, insulin. Insulin is secreted from β-islet cells to increase cellular glucose uptake. Long and short term changes in glucose availability and subsequent insulin demand require plasticity in the β-cells. This demand is placed on the ER for maintaining efficient levels of insulin peptide for secretion. Any changes in the UPR homeostasis mechanism may lead to dysfunction. Wolcott-Rallison syndrome is a condition in which sufferers develop type 1 diabetes, growth retardation and neurological disorders (Iyer et al., 2004). It has been mapped to mutations in the PERK gene (Delepine et al., 2000). The inability of PERK to function as an activator of translational repression leads to a continual translation of peptides despite the inability to fold them. This activates apoptosis and leads to β-cell death and diabetes (Zhang et al., 2002). Interestingly mice with eIF2a S51A mutations, the phosphorylation site in response to stress, also show diabetic phenotypes on a high fat diet. Homozygous mice die in embryogenesis due to β-cell deficiency whilst heterozygotes are prone to obesity and developing type 2 diabetes (Scheuner et al., 2001; Scheuner et al., 2005).In addition UPR markers have been shown to be upregulated in obese, type 2 diabetic patients (Boden et al., 2008).

Insulin resistance may be mediated through converging pathways with ER stress. Insulin receptor (IRS1) gets phosphorylated by JNK (Aguirre et al., 2000; Lee et al., 2003). JNK is a target of IRE1 mediated activity on ER stress (Urano et al., 2000). Hyperphosphorylated PERK and eIF2α have been found in the liver and adipose tissue of obese mice, and have been shown to induce JNK activation (Ozcan et al., 2004). Activation of the JNK pathway increases insulin resistance. XBP1 deficient mice develop similar phenotype to type 2 diabetes. These findings implement the UPR in the development of Type 1 and 2 diabetes and a link to obesity.

Seipinopathy describes a range of neurological disorders caused by the misfolded, ER resident, glycoprotein Seipin. Seipin is a transmembrane protein of unknown function (Lundin et al., 2006). Mutations in the N-glycosylation regions of Seipin can lead to misfolding and aggregation (Windpassinger et al., 2004). ER stress may be the process which eventually leads to neuronal cell death. Mutant protein expression induces apoptosis, and expression of mutant Seipin causes upregulates UPR markers (Ito and Suzuki, 2007). They also form cell inclusion bodies in vitro (Ito et al., 2008), although this has not been demonstrated in vivo.

4.1.5 The UPR in *C. elegans*

Although the components and regulation of the unfolded protein response remain highly conserved between organisms, there are differences that are specific to *C. elegans* and processes that have been further examined using this model organism. These extra details are addressed in the following sections. An overview of the *C. elegans* UPR is given below (Figure 4.2).

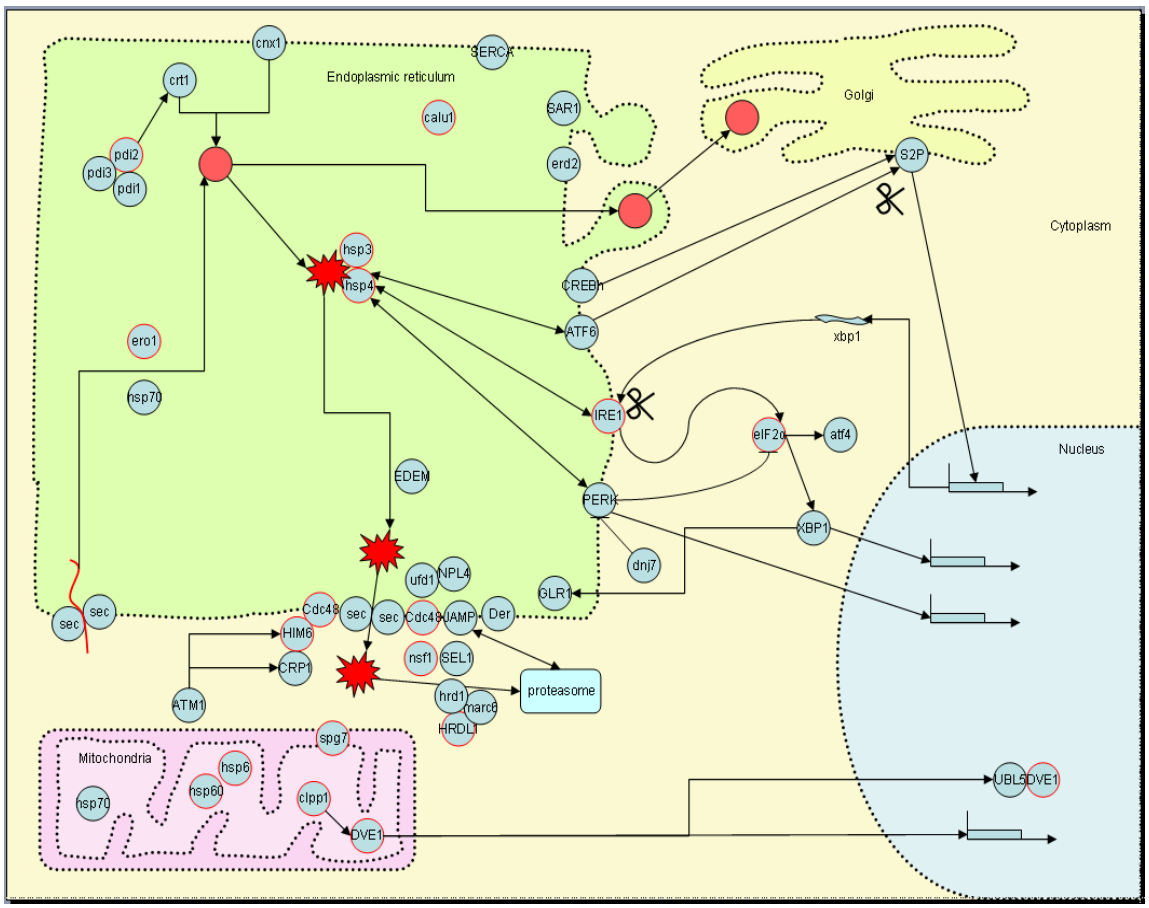


Figure 4.2. The UPR network of protein interactions in *C. elegans*.
Schematic network representation of the events controlling the UPR based on evidence from *C. elegans* studies. Proteins given in blue circles with black (non-lethal on KO) or red (lethal on KO) outline. Mitochondria highlighted in pink, nucleus in light blue, Golgi in yellow and ER in green. Dotted line representative of membrane lipid bilayer. Arrows indicative of interaction events. Blue rectangles in nucleus indicate transcription events. Scissors indicate splicing or modification event.

4.1.6 Endoplasmic Reticulum-Associated Degradation (ERAD)

On activation of the UPR one of the initial responses is to remove and recycle the offending misfolded proteins from the ER. The process of ERAD works to remove proteins from the ER by retrotranslocation before targeted destruction by the proteasome. Misfolded proteins are targeted by chaperones and localised to the ER membrane where SEC61 and accessory proteins, such as Derlin and p97, can transport the peptide out by retrotranslocation (Vembar and Brodsky, 2008). Removal by SEC61 initiates targeted ubiquitination by membrane bound proteins such as gp78, parkin, RNF5/RMA1 and HRD1.

The ERAD protein JNK-associated protein (JAMP) promotes proteasome localisation to the ER and links the removal of misfolded proteins to their subsequent destruction (Tcherpakov et al., 2008). JAMP is a membrane bound protein with its N-terminal in the ER lumen interacting with ERAD components, whereas the C-terminal remains cytosolic, associating with proteasome subunits RPT4 & RPT6. JAMP associates with proteasomal subunits during both normal and stressed conditions. Proteasome binding is negatively regulated by RNF5 ubiquitination of JAMP (Tcherpakov et al., 2009). In addition JAMP interacts with JNK and

modulates its activity (Kadoya et al., 2005). JAMP mutants have delayed development on exposure to ER stressors and are hyper sensitive to tunicamycin. Mutants also show higher basal levels of ER stress; similar to *Sel1*, *Derlin* and *Ero1* mutants (Tcherpakov et al., 2008).

When misfolded proteins are retrotranslocated from the ER via SEC61 they are subjected to ubiquitination, ready for proteasomal degradation (Kostova et al., 2007). *C. elegans* contain three E3 ubiquitin ligases HRD1, HRDL-1 and MARC-6. These are transmembrane, ring finger proteins that share homology to ubiquitin ligases in yeast and humans. HRD1, HRDL-1 and MARC-6 double and single mutants affect growth and share phenotypes that suggest a role in protein folding. It is believed that these proteins play a role in ERAD mediated ubiquitination (Sasagawa et al., 2007). Of the E3 ligases only HRD1 interacts with HSP-3 and HSP-4 (*C. elegans* homolog of BiP) and p97. *C. elegans* contains two homologs of p97 (cdc-48.1 & cdc-48.2). p97 forms a homo-hexamer pore at which its N-terminal domain interacts with regulatory proteins (Mouysset et al., 2006). The interaction between p97, BiP and HRD1 may help coordinate the protein removal with targeted destruction, although the role of the homologs in this process remains unclear. These interactions may be of specific importance during development, HSP-3 is involved in intestinal development and HSP-4 in gonad formation (Sasagawa et al., 2007)

4.1.6.1 UPR Stress Responses in *C. elegans*

The *ire-1:xbp-1* arm of the UPR is consistently needed to activate response genes to stress and can be seen in *C. elegans* to be one of the main, yet remarkably diverse transcriptional activators. Shen *et al*. looked at the changes in mRNA in UPR mutants, identifying 202 genes, of which 84% of these genes regulated by *ire-1* and *xbp-1* (Shen et al., 2005). Interestingly the same paper identifies a novel bZIP transcription factor CREBh, which shares homology with CREB and ATF family members, under the control of the *ire-1:xbp-1* arm. The role of CREBh in response to unfolded proteins is of interest and has been discussed earlier.

Calreticulin is a calcium ion binding protein localised in the ER and which is essential for normal physiological conditions. Calreticulin is upregulated on heat stress and 7% ethanol treatment (Park et al., 2001). Calreticulin functions to maintain protein folding and calcium homeostasis in the ER, and its function is partially redundant with calnexin (Lee et al., 2005). Calreticulin (*crt-1)* mutants are hypersensitive to tunicamycin, and CRT1 is induced by both tunicamycin and heat stress. The promoter region of *crt-1* contains two XBP1 binding sites and an ERSE sequence. RNAi analysis suggests that *crt-1* regulates its own expression in a negative feedback mechanism, and in addition regulates levels of *pdi-2* and *pdi-3*, disulfide bond forming chaperones (Lee et al., 2007; Lee et al., 2006).

Calcium levels are an important factor in ER homeostasis. Store operated calcium entry (SOCE) is activated on ER calcium depletion and is an essential to refill ER calcium stores and

aide calcium signalling. Human STIM1 and STIM2 have been identified as mediators SOCE and function to differentially modulate calcium channels (Liou et al., 2005; Soboloff et al., 2006). A STIM1 homolog is present in *C. elegans*. Efflux of calcium out of the ER can trigger the UPR as well as RNAi of calcium pumps that restore ER calcium levels. Knockout of STIM1 does not trigger the UPR and is unlikely to play a role in refilling the ER calcium stores (Yan et al., 2006).

Small GTP binding proteins of the RAS super-family may also have a role in ER stress. Knockout of SAR1 by RNAi induces the UPR. CDC42 and CRP1 are required for UPR activation as RNAi knockdown leads to reduced levels of ER stress on tunicamycin treatment (Caruso et al., 2008). CDC42 has been implemented in Golgi to ER vesicle trafficking, whilst CRP1 has a role in membrane trafficking in epithelial cells (Jenna et al., 2005; Luna et al., 2002). Interaction analysis for CRP-1 using GST pull downs with mass spectrometry identification revealed interactions with *C. elegans* p97 (CDC48.1/2) ATM1, HIM6 (BLM homolog) and WRN. This suggests that the function of CRP-1 might be involved in transcriptional activation through chromatin remodelling. Mutants in *crp-1, atm-1* and *cdc-48.1* all show hyper-sensitivity to tunicamycin (Caruso et al., 2008).

The UPR may not be limited to purely ER mediated activation, and may be triggered separately in the mitochondria (mtUPR). The mitochondrial stress response activates genes responsible for chaperone and folding proteins independent from ER stress response (Zhao et al., 2002). Mitochondrial stress is regulated separately from ER stress by the AP-1 promoter (Aldridge et al., 2007). This stress response is also present in *C. elegans* (Yoneda et al., 2004). DVE1 and UBL5 are found in the nucleus of mitochondria rich cell types and are required for the activation of the UPR independently of ER stress. The *C. elegans* ClpP homolog (*clpp-1*) controls the translocation of DVE1 to the nucleus independently of UBL-5. *clpp-1* RNAi knockout stops activation of UBL-5 and subsequent DVE1/UBL5 complex formation (Haynes et al., 2007). In bacteria ClpP is responsible for proteolysis of a repressor of the stress response (Frees et al., 2007), and it is possible that this mechanism may have been conserved in eukaryotic mitochondria. The activation of DVE1 and UBL5 by ClpP may represent a signal transduction pathway for mitochondrial protein stress.

4.1.6.2 UPR influences on behaviour

It is not currently clear whether the UPR influences or outputs any behavioural response, although it appears that perturbed pathways that activate the UPR may lead to behavioural changes.

Hereditary spastic paraplegia (HSP) is an inherited neurological disorder characterised by spasticity in the lower limbs. The NIPA1 gene is implicated in HSP onset and disease progression (Rainier et al., 2003). The *C. elegans* homolog of NIPA1 shares ~45% identity with

the human homolog and, importantly, shares the disease causing site giving rise to the HSP phenotype in humans (Zhao et al., 2008). Using mouse cells Zhao et al. were able to show mutant NIPA1 retention in the ER due to protein misfolding. Expression of mutant NIPA1 in *C. elegans* gave a phenotype comparable to human HSP. Mutant worms were fully paralysed by day 9, with paralysis progressing from the posterior to anterior. Expression of mutant NIPA1 in *xbp-1*$^{-/-}$ background produced a complete rescue of paralysis and of longevity, indicating a role of the UPR in the progression of this disease.

AMPA-type glutamate receptors are one of the main types of excitory signalling components in the CNS. The function of these receptors relies on the correct subunit assembly and processing, which is mediated by the ER (Greger et al., 2007). Neurons in *C. elegans* require the UPR components to move receptor subunits through the ER.

ire-1/xbp-1 mutations are associated with a defective localisation of GLR-1 resulting in increased ER retention (Shim et al., 2004). GLR-1 is required for mechanosensory neurons (Maricq et al., 1995), and *ire-1/xbp-1* mutants are deficient in nose touch response in much the same way as *glr-1* mutants (Shim et al., 2004). This supports the hypothesis that the UPR can impact processing and expression of major determinants of neuronal function and has the potential to respond to a modified environment and/or pharmacological inputs.

4.1.7 Ethanol and the UPR

4.1.7.1 Acute effects

Ethanol is believed to be an inducer of endoplasmic reticulum stress and a cause of the UPR. It is unknown whether ethanol directly causes the UPR or triggers it as a secondary consequence of differential targets.

Several studies have shown HSP4 (GRP78) the sensor of unfolded protein in the ER and an indicator of UPR activation are upregulated in response to ethanol treatment. These studies have monitored transcript expression increases on ethanol treatment. NG108-15 neuroblastoma x glioma cells grown in the presence of 100mM ethanol for 24 hours caused a 2-fold increase in the abundance of *hsp-4* mRNA (Miles et al., 1994). In a follow up study the group shows that ethanol also potentiates an upregulation of *hsp-4* mRNA by thapsigargin, tunicamycin and brefeldin A (Hsieh et al., 1996). Increases in *hsp-4* mRNA after 100mM ethanol treatment were seen at 24h in SH-SY5Y cells, and in week 1 of an 8 week chronic treatment protocol. This increase was not observed at a protein level on acute and chronic ethanol treatment (Muhlbauer and Rommelspacher, 2003). Ethanol treatment of SH-SY5Y cells with 400 mg/dl over 12 hours showed no increase in ER stress proteins such as HSP4 and CHOP. Ethanol could however potentiate an ER stress response to tunicamycin and thapsigargin treatment by means of ROS production. Potentiation by ethanol could be lost on co-treatment with anti-oxidants (Chen et al.,

2008). Increases in both *hsp-4* mRNA and protein were observed in the liver and brain of rats fed a 5g / kg diet, although this was not observed in rats fed 2g / kg diet (Tunici et al., 1999). The upregulation of HSP4 a marker for ER stress and UPR induction is likely to be induced with chronic, high concentration ethanol treatment. The mechanism of induction may be translationally regulated as whilst *hsp-4* mRNA is upregulated on ethanol treatment, this affect is only seen at a protein level on high concentration and chronic treatment.

Chronic ethanol use has been implicated in the pathology of several diseases through activation of an unfolded protein response. Hyperhomocysteinemia is a condition of increased serum homocysteine and has been shown to trigger the UPR on chronic alcohol feeding (Kaplowitz and Ji, 2006). Hyperhomocysteinemia is a risk factor in a number of disorders such as atherosclerosis and hepatosteatosis. Hyperhomocysteinemia is believed to play a role in lipid dysregulation through the protein SREBP. Triggering the UPR hyperhomocysteinemia activates the cleavage of SREBP through S1P and S2P, similar to ATF6. Ethanol is believed to increase homocysteine levels by down regulating methionine synthase. Ethanol mediated liver toxicity and steatosis can be prevented by supplementation of diet with betaine (Esfandiari et al., 2005). The conversion of homocysteine to adenosylmethionine by methionine synthase is a folate dependent process. ER stress may also be exacerbated by the activation of CYP2E and oxidative stress through ethanol metabolism and ROS generation.

4.1.7.2 UPR, ethanol and *C. elegans*

Ethanol has also been shown to trigger the unfolded protein response in *C. elegans*. Using a concentration of 7% ethanol (1.4 M) *C. elegans* is completely paralysed until recovery on treatment withdrawal, from which 50% of worms survive. Using 7% ethanol microarray data revealed that several groups of transcripts were upregulated; amongst them were several indicators of an unfolded protein response (Kwon et al., 2004). It has also been shown that 7% ethanol at 16°C for 6 hours is able to trigger an unfolded protein response using the reporter system *phsp-4::GFP* (Kwon et al., 2004).

The unfolded protein response is implicated in a range of disorders and is activated on a number of stresses. The unfolded protein response is an evolutionary conserved mechanism for coping with stress which extends to the model organism *C. elegans*. The effects of ethanol on *C. elegans*, discussed in Chapter 1, exhibit differential behavioural responses depending on the method of conditioning. These are both time and dose dependent, with the severity of the behavioural response increasing with dose and time conditioned. Ethanol has been shown to induce a stress response both in mammals and in *C. elegans* (Kaplowitz and Ji, 2006; Pandol et al., 2010; Uccelletti et al., 2004). The aim of this research is to investigate whether the ethanol conditioning paradigms used to elicit a behavioural response also activate physiological stress response. If a stress response is activated whether this modulates the behavioural response to ethanol in *C. elegans*.

4.1.7.3 Motivation for current work

Provoked by published information and our own proteomic investigation pointing to a modified ER response during ethanol conditioning led us to investigate using the *hsp-4* (BiP/GRP78) promoter linked GFP reporter array in *C. elegans* as a mechanism for monitoring. In addition, we took advantage of the established behavioural assays to investigate if the hsp-4 induced pathways are involved in the behavioural consequence of withdrawal. This allowed us to integrate aspects of existing data to test potential for new pathways to be involved in ethanol induced behaviours.

4.2 Methods

4.2.1 Nematode strains.

As detailed in **2.2.2.2**

4.2.2 Worm preparation.

As detailed in **2.2.2.3**

4.2.3 Escherichia coli OP50 culture.

As detailed in **2.2.2.1**

4.2.4 Worm race assay.

As detailed in **2.2.3.1**

4.2.5 Ethanol test.

As detailed in **2.2.2.4**

4.2.6 UPR assay.

Culture plates (3cm) hand poured with 4.5 ml of NGM agar and left 24h to set. Tunicamycin (Sigma-Aldrich) was diluted to 100x solution in DMSO before being added to molten NGM media plates to give a final concentration of 5 μg ml^{-1} or 30 μg ml^{-1}. Comparative amounts of DMSO (0.01%) were added to molten NGM control plates. Dried plates were seeded with 50 μl of *Escherichia coli* OP50, standard nematode food, at 0.8A [OD_{600}] and left 24h. Selected seeded plates were treated with ethanol (99.9%) to give final concentrations as indicated. Plates were left 24h for the ethanol to equilibrate across the agar. Larval stage 4, *zdIs4* worms were separated 1 day before treatment and incubated 24h at 20°C. L4+1 day worms were transferred to treatment plates for 24 hours. Images were recorded using a CCD camera mounted to a fluorescent microscope at described intervals. Image contrast was normalised to tunicamycin, GFP positive samples.

4.2.7 GFP fluorescence in vivo.

6cm plates were seeded with 50 µl of *E. coli* OP50 at 0.8A [OD_{600}] and left 24h to settle. Plates were treated with either M9 buffer 100ml, Ethanol [250mM] or iso-propanol [250mM] at a plate ratio of 4:1:1 respectively. Larval stage 4, *pmyo2::GFP (slo-1 neuronal rescue)* worms were separated 1 day before treatment and incubated 24h at 20°C. L4+1 day worms were transferred to M9 plates for 5 min. Images were recorded using a CCD camera mounted to a fluorescent microscope after 5 min. Worms were then transferred to test plates of either M9, ethanol or iso-propanol for 5 min before photographing.

4.2.8 eGFP fluorescence in vitro.

EtOH solutions of various concentrations were made by serial dilution in MQ H_2O. The different solutions of EtOH were added 1:1 to a solution of soluble eGFP (purified from GST-eGFP diluted in MQ H_2O) in a 1.5ml reaction tube. Three 100µl aliquots of this were transferred to the wells of a black-walled, clear-bottomed 96-well plate. The plate was read in a Tecan-Sapphire fluorescence plate reader using FITC excitation/Emission settings (Ex485, Em535, 20nm bandwidth). Each point is the mean of the three wells. The 1st scan (black line) was done initially @ 15:56, the other scans were made subsequent to this (@ 16:47, 16:49 and 16:50 respectively) using the same plate-reader settings after pipetting out the 2nd experiment (the plate was not kept in the dark). These were provided by Tom Carter and Matthew Hannah, Molecular Neuroendocrinology, Mill Hill.

4.3 Results

Ethanol has been shown to be a source of ER stress in both mammals and *C. elegans*. Unfolded protein response was monitored in *C. elegans* using the feedback mechanism of HSP4/BiP induction on ER stress. This was done using a GFP tagged HSP4 protein ER stress increases HSP4 protein levels and the level of fluorescence in vivo. This line is an integration of a HSP-4 promoter that drives expression of GFP upon ER production. Under control conditions the level of expression in well fed worms is too low to produce a detectable signal. In contrast growing worms on plates laced with tunicamycin shows a rapid and sustained induction of expression. These preliminary observations indicate that this reporter can be used to investigate if ER stress (see Figure 4.3 for example worms).

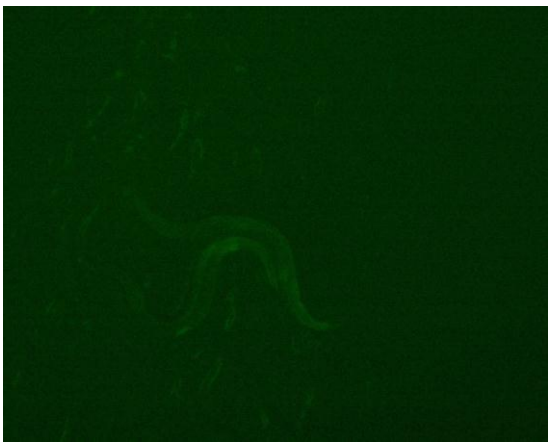
4.3.1 Treatment of worms with ethanol show no induction of an UPR

Fifty larval stage 4 worms + 1day worms were treated with tunicamycin 30 µg ml^{-1} positive control ethanol 450mM, DMSO vehicle control and no treatment for 24 hours and then withdrawn from treatment. Treatment with the N-glycosylation inhibitor Tunicamycin at 30 µg ml^{-1} activates increased GFP fluorescence compared to the basal levels of no treatment and DMSO vehicle controls. Ethanol intoxication showed no detectable increase in fluorescence

activate the unfolded protein response above the basal levels of controls at the 6 (Fig 4.3 (A)) and 24 h time limits (Fig 4.3 (B)). Ethanol treatment also fails to induce a response on treatment withdrawal (Fig 4.3 (C)). Tunicamycin treatment does induce or retain a persistent UPR on withdrawal from the pharmacological insult.

Ethanol intoxication shows a behavioural phenotype (Figure 4.4). This is manifest in the withdrawal response when the worms were removed from ethanol and tested in a food race. The removal from treatment by ethanol, tunicamycin and controls continue to show a behavioural phenotype consistent with expected results when assayed in a food race paradigm. Control Worms show 87% and 81% of the population reaching the food after a 2 hour period respectively consistent with previous data (see chapter 2). In contrast worms treated with ethanol for 24h and then withdrawn show 17% of the population reaching the food reward after 2 hours. Tunicamycin treated worms also show a deficit in the percentage of the population reaching the food when subsequently tested in the food race in the absence of tunicamycin after 2 hours at 46%. This is less than control worms but better than the ethanol withdrawn worms. This suggests that the worms under unfolded protein stress may exhibit a behavioural phenotype.

A



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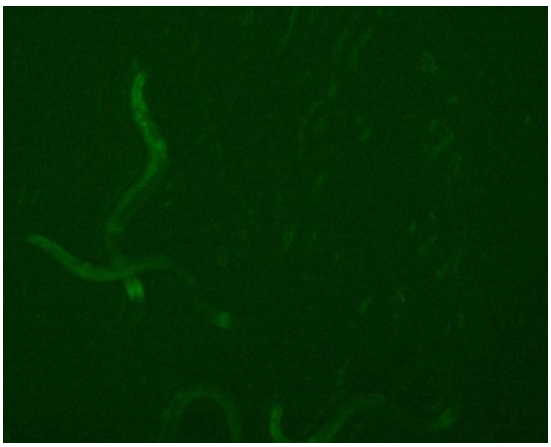

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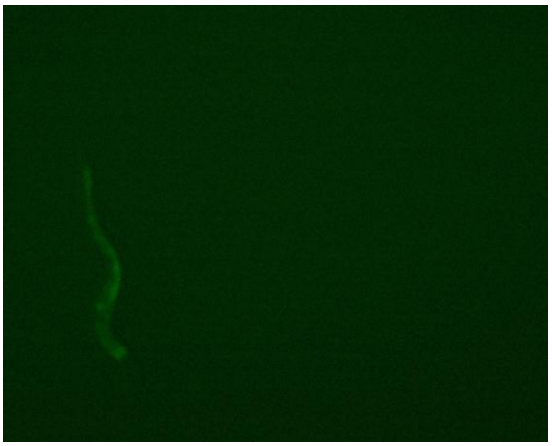
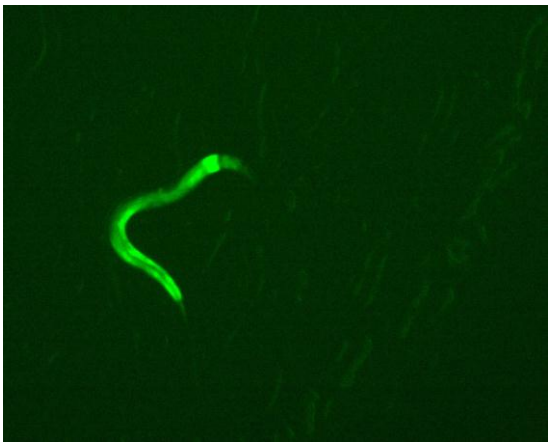
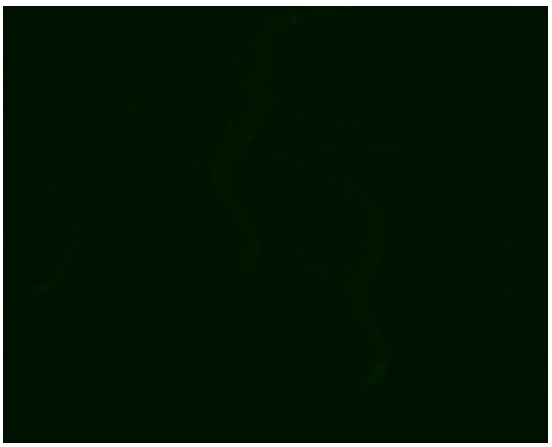


ETHANOL



TUNICAMYCIN

B



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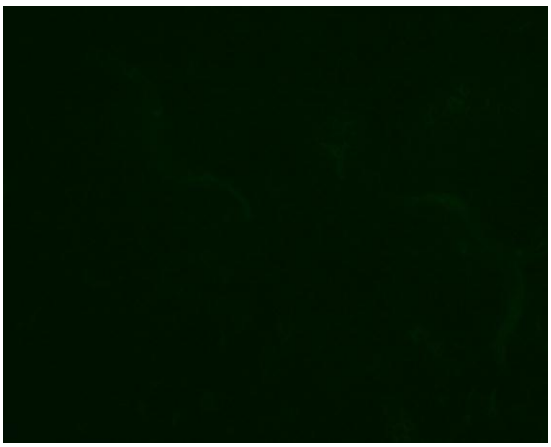

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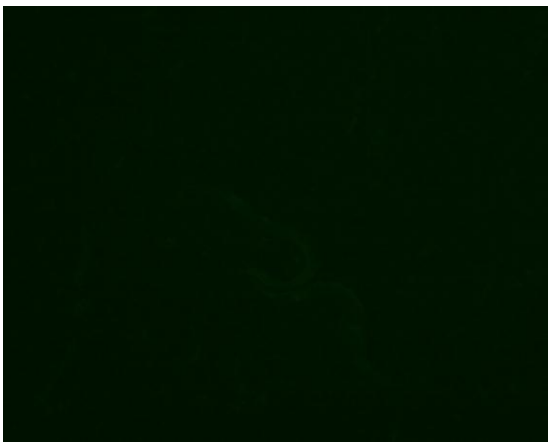

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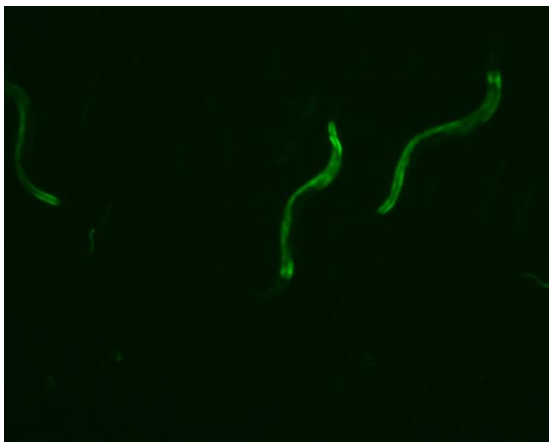


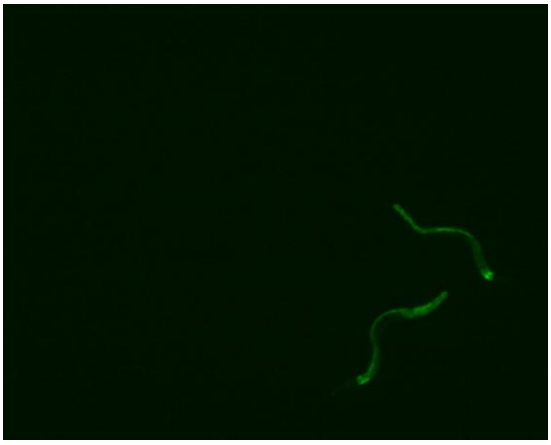
TUNICAMYCIN

C

CONTROL

DMSO

ETHANOL



TUNICAMYCIN

Figure 4.3. *zdIs4* (*phsp4::GFP*) worms after ethanol conditioning at 6 hours, 24 hours and 1 hour post treatment withdrawal.
***zdIs4* (*phsp4::GFP*) worms photographed under 484 nm fluorescent light at 35x magnification. (A) Worms at 6 hours after treatment with ethanol 450mM (EtOH), tunicamycin 30 µg ml^{-1} (TUN), DMSO 0.01% (DMSO) and no treatment (Control). (B) Worms at 24 hours after treatment with ethanol 450mM (EtOH), tunicamycin 30 µg ml^{-1} (TUN), DMSO 0.01% (DMSO) and no treatment (Control). (C) Worms at 1 hour 10 min post 24 hour treatment with after treatment with ethanol 450mM (EtOH), tunicamycin 30 µg ml^{-1} (TUN), DMSO 0.01% (DMSO) and no treatment (Control).**

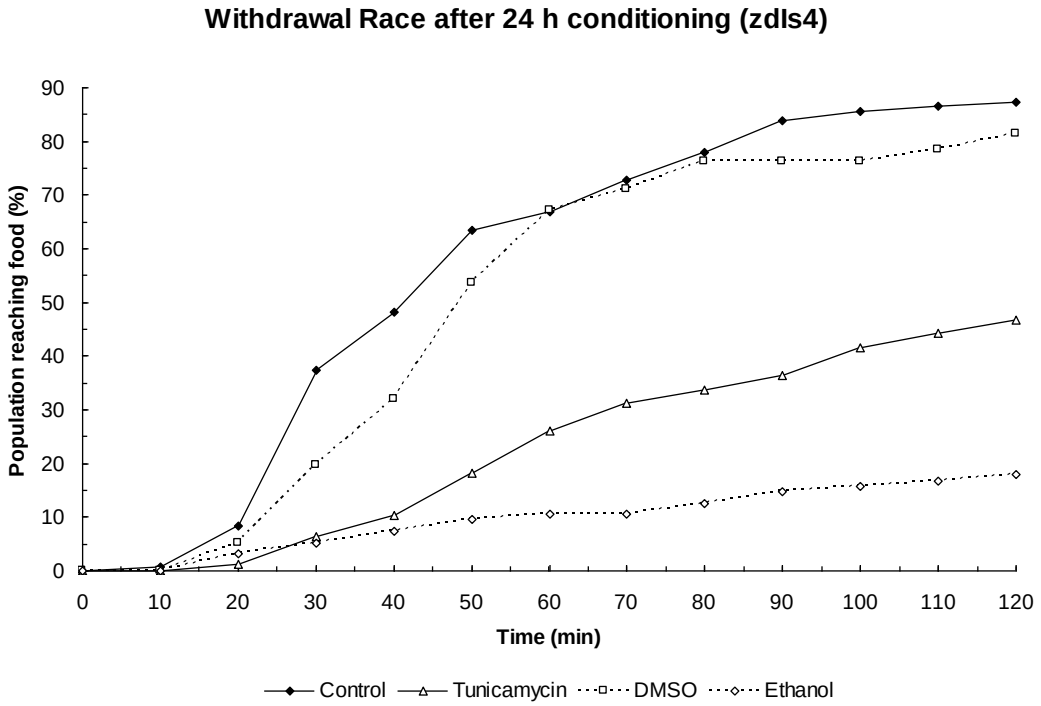


Figure 4.4. *zdIs4* (*phsp-4::GFP*) worms raced over 5 cm towards a food reward. Population reaching food is recorded every 10 min for 2 hours. Worms conditioned for 24 hours and withdrawn from treatment of: ethanol 450mM, tunicamycin 30 µg ml^{-1}, DMSO (0.01%) and no treatment (control) before race. 87% untreated worm population, 81% DMSO treated worms, 46% tunicamycin treated worms and 17% ethanol treated worms reached the food reward after 2 hours.

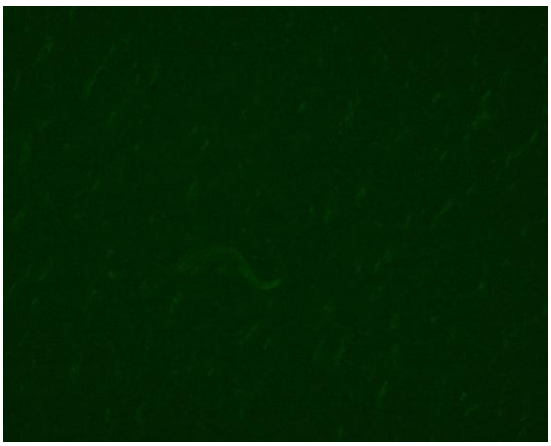
4.3.2 Treatment of worms with ethanol and tunicamycin does not potentiate an UPR

Initial results suggested that the addition of ethanol to *C. elegans* at 450mM was insufficient to trigger an unfolded protein response (Fig 4.3). It was investigated whether ethanol was enough to prime a stress response on dual treatment with tunicamycin. It was also investigated whether ethanol at higher concentrations than previously used was enough to trigger an unfolded protein response.

20 larval stage 4 + 1 day worms were treated with either ethanol (450mM, 900mM, 1.35M) ethanol (450mM)and tunicamycin (5 µg ml^{-1}) or a DMSO vehicle control, DMSO. The worms were investigated for fluorescence after 6 hours (Fig 4.5) and 24 hours (Fig 4.6). Ethanol at 450mM alone was unable to induce a GFP positive stress response beyond basal levels of controls after 6 hours (Fig 4.5 (A)) and 24 hours (Fig 4.6 (A)). Treatment with ethanol and tunicamycin showed no increased GFP linked stress response beyond controls at both 6 hours (Fig 4.5 (C)) and 24 hours (Fig 4.6 (C)). This suggests that ethanol is unable to potentiate a stress response by tunicamycin treatment for 24 hours.

Treatment of worms with increased ethanol concentrations 900mM and 1.35M showed no HSP-4 induction at the 6 hour time period (Fig 4.5 (B)). At 24 hours 1.35M ethanol may induce a slight increase in GFP and may indicate a low level stress response (Fig 4.6 (B)).

A



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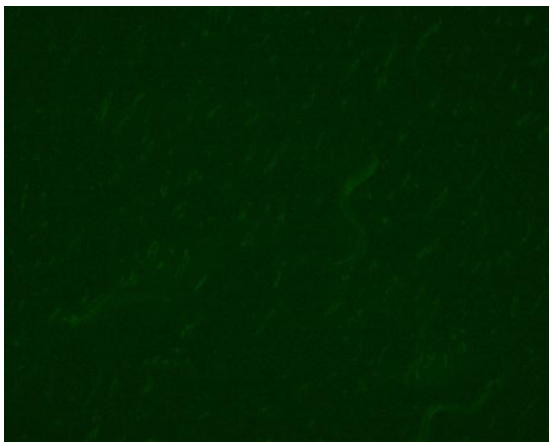

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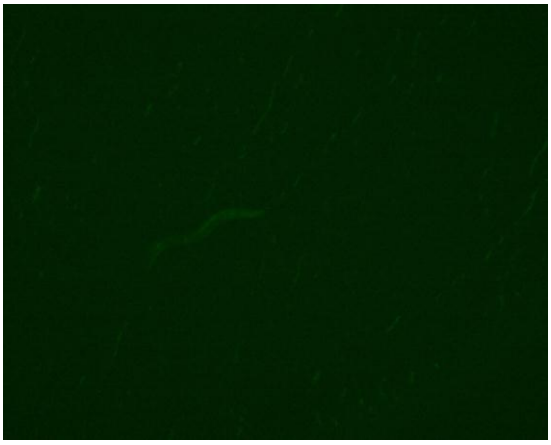
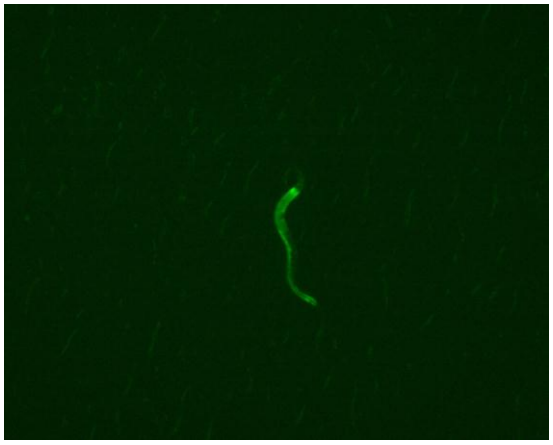
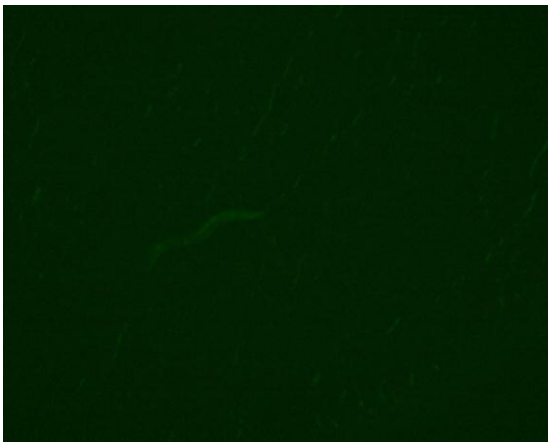


ETHANOL

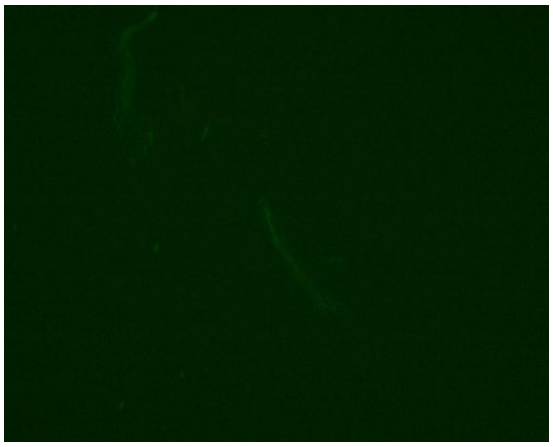


TUNICAMYCIN

B



ETHANOL 450mM



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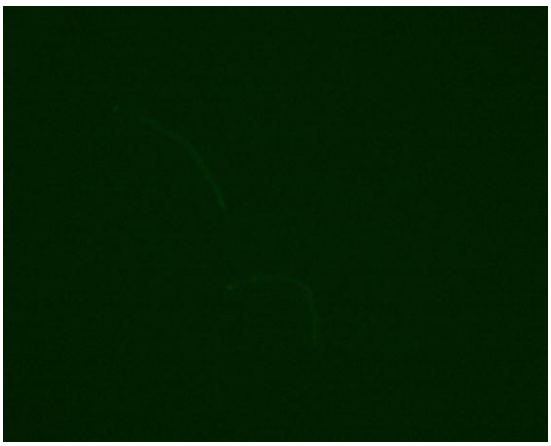

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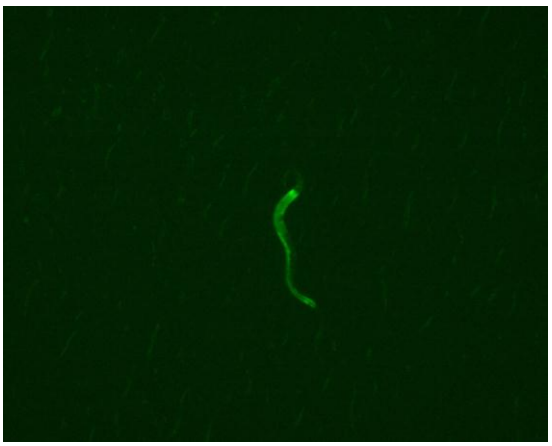
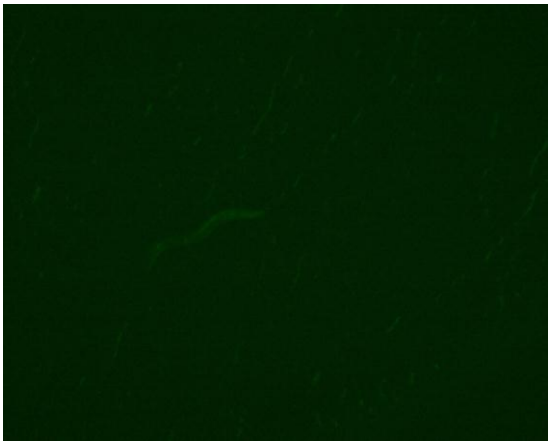


TUNICAMYCIN

C



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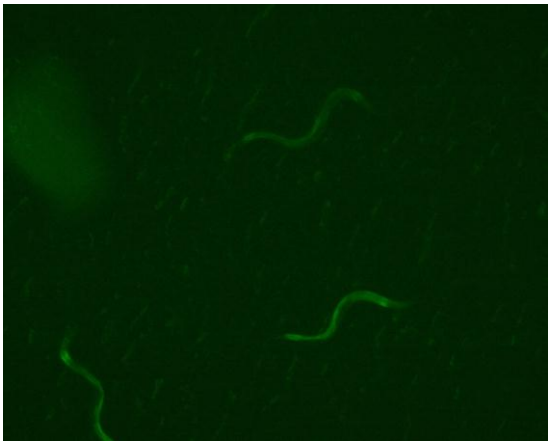
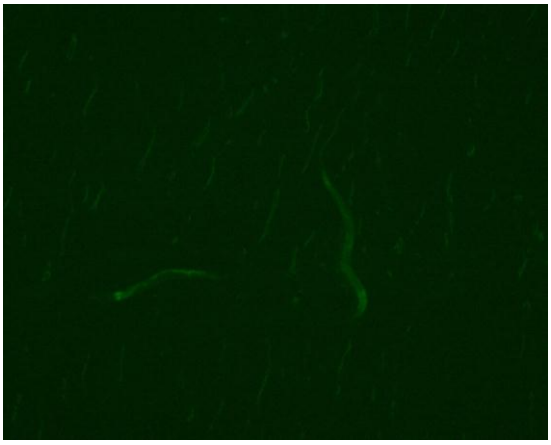
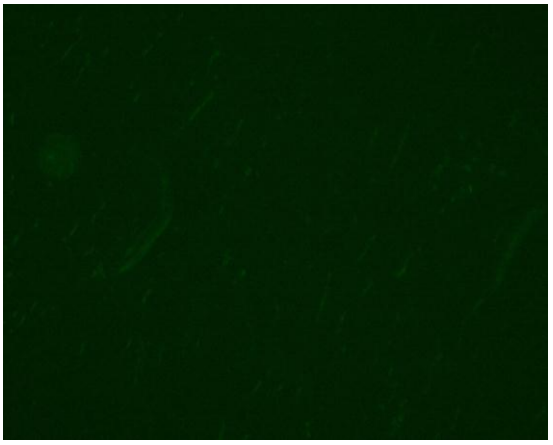


TUNICAMYCIN 5



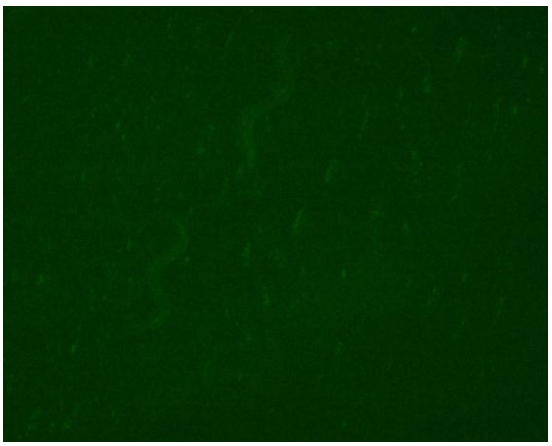
ETHANOL & TUNICAMYCIN



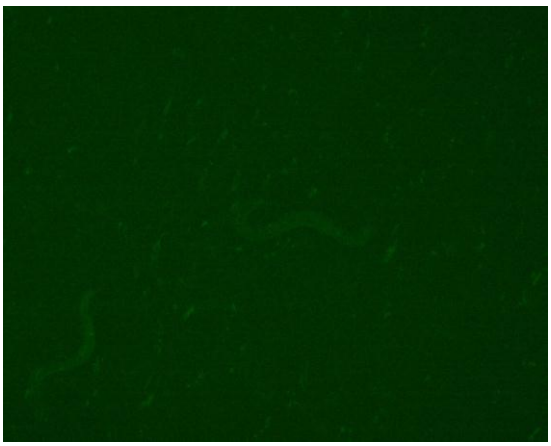
ETHANOL & DMSO

Figure 4.5. *zdIs4* (*phsp4::GFP*) worms after conditioning for 6 hours with increasing ethanol concentrations and dual tunicamycin treatment.
***zdIs4* (*phsp4::GFP*) worms imaged under 484 nm fluorescent light at 35x magnification. (A) Worms at 6 hours after treatment with ethanol 450mM (EtOH), tunicamycin 30 µg ml^{-1} (TUN), DMSO 0.01% (DMSO) and no treatment (Control). (B) Worms at 6 hours after treatment with ethanol 450mM (EtOH), 900mM (EtOHx2), 1.35M (EtOHx3) and tunicamycin 30 µg ml^{-1} (TUN). (C) Worms at 6 hours after treatment with ethanol 450mM (EtOH), tunicamycin 5 µg ml^{-1} (TUN), ethanol 450mM and tunicamycin 5 µg ml^{-1} (EnT), ethanol 450mM and DMSO 0.01% (EnD).**

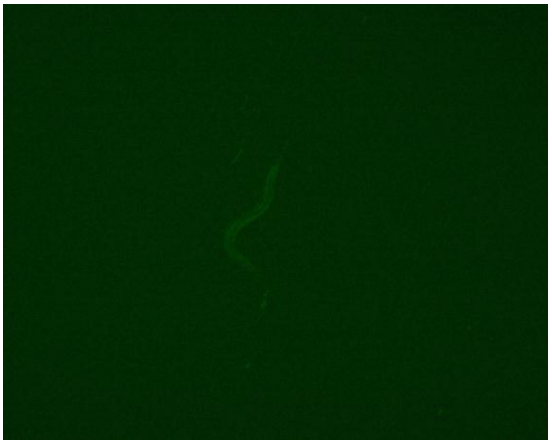
A



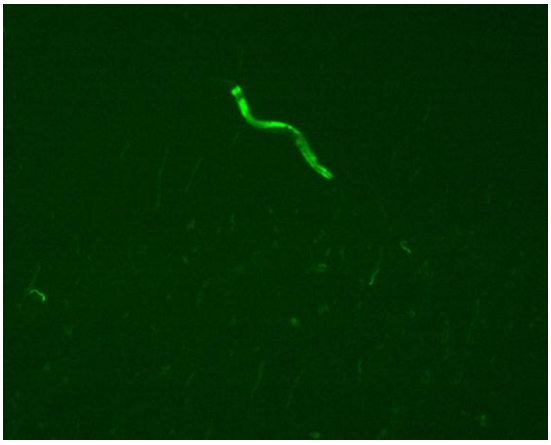
CONTROL



DMSO

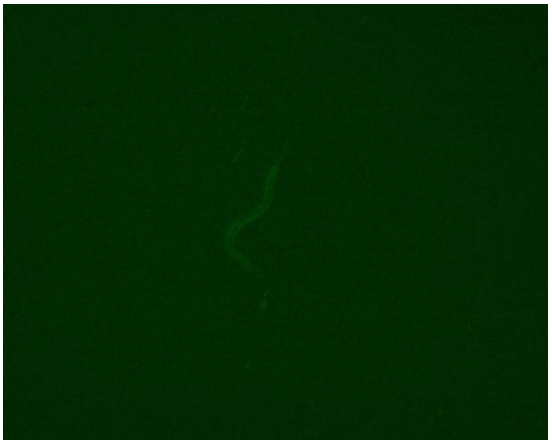


ETHANOL



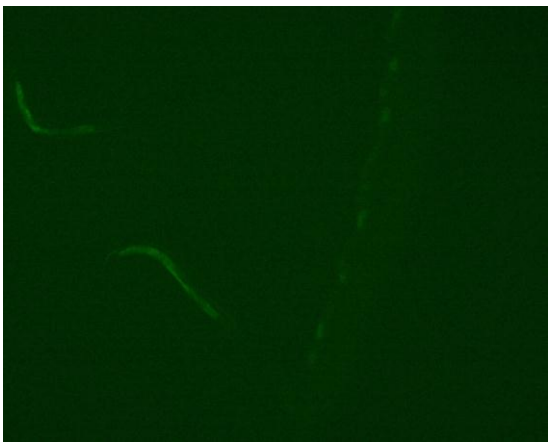
TUNICAMYCIN

B



ETHANOL 450mM

ETHANOL 900mM



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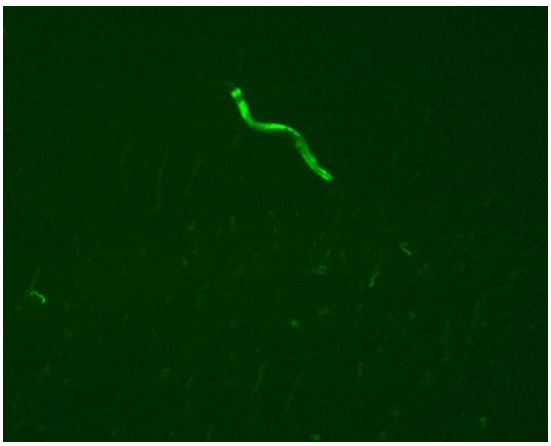
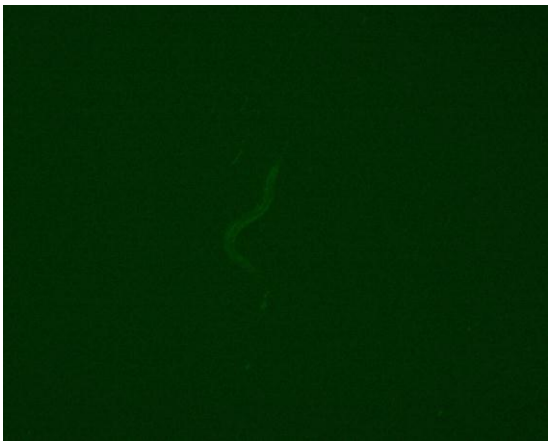
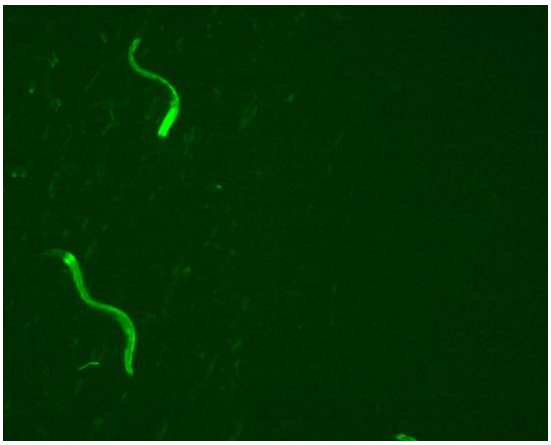


TUNICAMYCIN

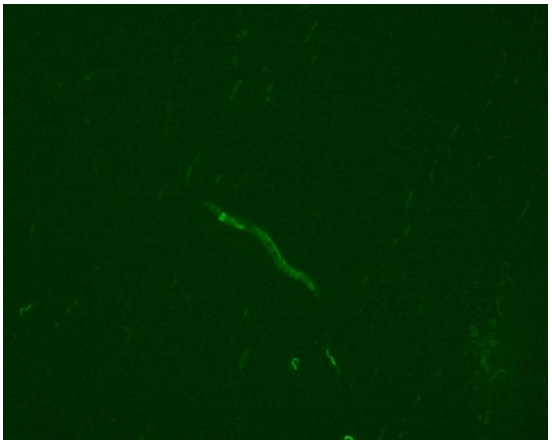
C



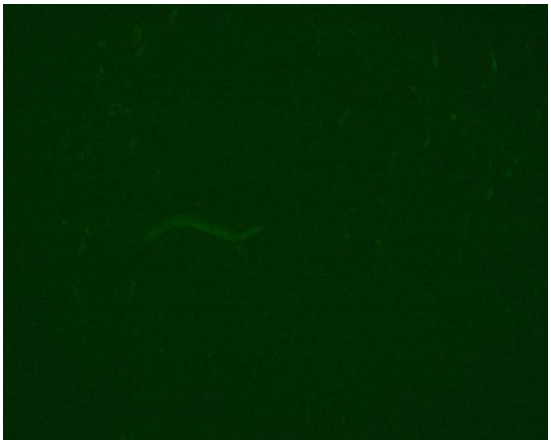
ETHANOL 450mM



TUNICAMYCIN 5



ETHNAOL & TUNICAMYCIN



ETHANOL & DMSO

Figure 4.6. *zdIs4* (*phsp4::GFP*) worms after conditioning for 24 hours with increasing ethanol concentrations and dual tunicamycin treatment.
***zdIs4* (*phsp4::GFP*) worms photographed under 484 nm fluorescent light at 35x magnification. (A) Worms at 24 hours after treatment with ethanol 450mM (EtOH), tunicamycin 30 µg ml^{-1} (TUN), DMSO 0.01% (DMSO) and no treatment (Control). (B) Worms at 24 hours after treatment with ethanol 450mM (EtOH), 900mM (EtOHx2), 1.35M (EtOHx3) and tunicamycin 30 µg ml^{-1} (TUN). (C) Worms at 24 hours after treatment with ethanol 450mM (EtOH), tunicamycin 5 µg ml^{-1} (TUN), ethanol 450mM and tunicamycin 5 µg ml^{-1} (EnT), ethanol 450mM and DMSO 0.01% (EnD). These results are representative images from three independent experiments with 20 worms per treatment.**

4.3.3 Investigating the time dependent effects of ethanol treatment on the UPR

Treatment with ethanol 450mM failed to produce an assayable unfolded protein response at the 6 hour and 24 hour time points (Fig 4.3). Treatment with ethanol and tunicamycin combined also failed to potentiate an unfolded protein response at 6 hours (Fig 4.5) and 24 hours (Fig 4.6). It was the investigated whether the UPR is induced on acute ethanol and ethanol and tunicamycin combined treatments over a 6 hour period (Fig 4.7).

L4 +1 worms were treated with ethanol 250mM, tunicamycin 5 μg ml^{-1}, DMSO vehicle control, heat shock (28°C), ethanol 250mM and tunicamycin 5 μg ml^{-1}, ethanol 250mM and DMSO 0.01% control and no treatment for 24 hours. Samples were photographed at 1 hour intervals for 6 hours (Fig 4.7) and then again at 24 hours and 1 hour post treatment withdrawal (Fig 4.8). Heat shock was stopped at 6 hours

Consistent with previous results ethanol showed no induction of GPR and, by extension, the UPR at 1 hour intervals for 6 hours, or at 24 hours. In addition there was no induction following a 1 hour post withdrawal. Tunicamycin showed an induction of GFP/UPR at 4 hours onwards. Ethanol and tunicamycin combined treatment also showed induction at 4 hours onwards and was indistinguishable from tunicamycin only treatment. Induction remained constant on withdrawal from treatment for both ethanol and tunicamycin and tunicamycin only samples.

Heat shock treatment induced the UPR from the 2 hour time point until the 6 hour as indicated by the relative increase in the organism's fluorescence. After removal from the heat shock the increased florescence had dissipated by the 24 hour time point. An induction of the UPR by heat shock treatment confirms that the strain is not specific for tunicamycin treatment only and that ethanol fails to induce an unfolded protein response and potentiate ER stress induced by tunicamycin.

Withdrawal from treatment induces a behavioural response in worms (Fig 4.9). 68% worms receiving no treatment (control) reached the food reward after 2 hours. Tunicamycin, DMSO and heat shock treated worms had slightly less of the population reaching the food reward at 51%, 54% and 54% respectively. This is in contrast to ethanol treated worms which only 7% of the population reached the food after 2 hours. Ethanol and DMSO combined treated worms had 4% of the population reach the food similar to ethanol only treatment. Worms treated with ethanol and tunicamycin treatment had no worms make the food reward after 2 hours which suggest that the reduced locomotory phenotype is additive and produced through distinct mechanisms.

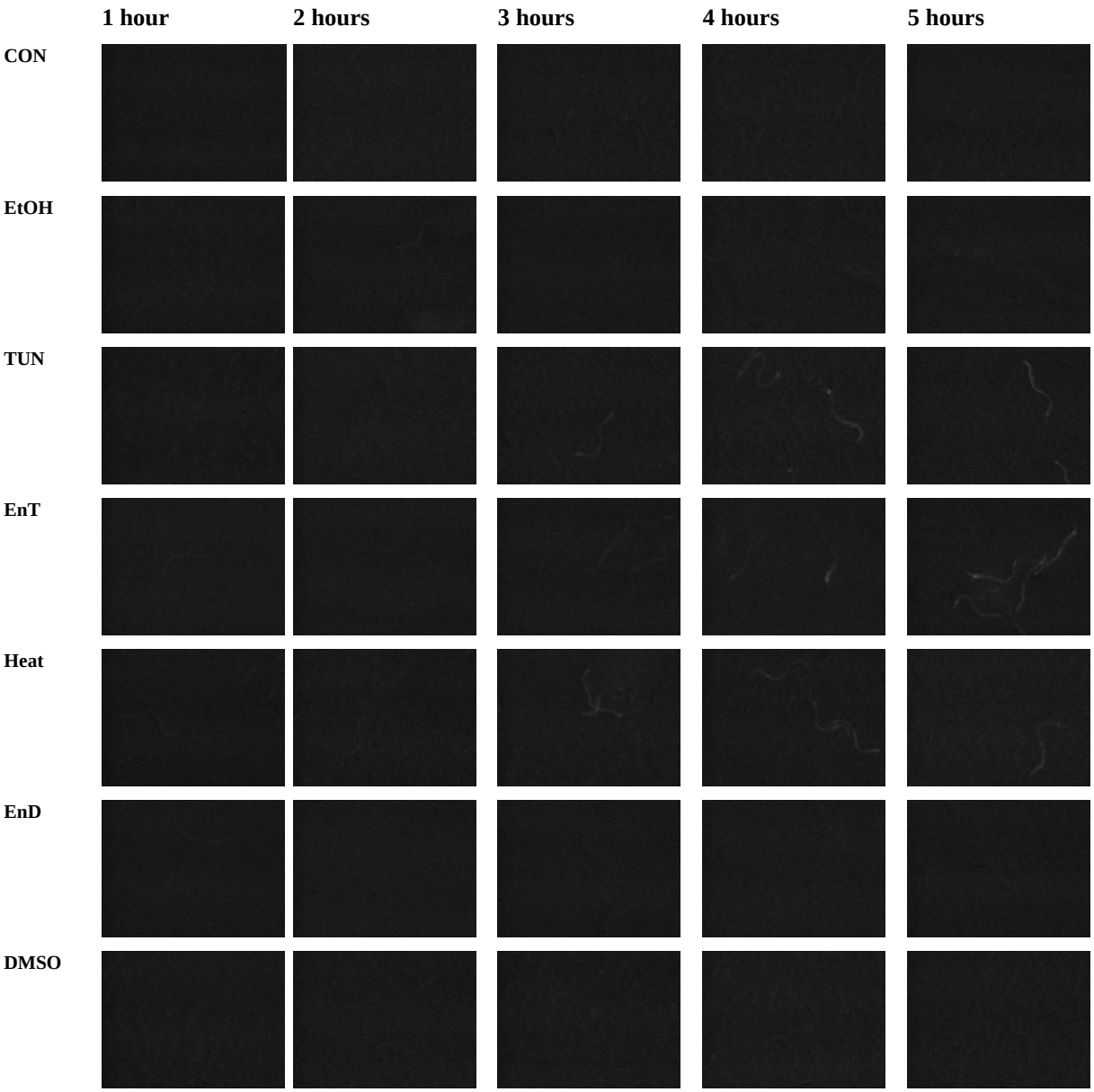


Figure 4.7. *zdIs4* (*phsp4::GFP*) worms at 1 hour intervals for 6 hours with ethanol and dual tunicamycin treatment.
***zdIs4* (*phsp4::GFP*) worms photographed under 484 nm fluorescent light at 35x magnification. Worms treated with ethanol 250mM (EtOH), tunicamycin 5 µg ml^{-1} (Tun), DMSO 0.01% (DMSO), ethanol 250mM and tunicamycin 5 µg ml^{-1} (EnT), ethanol 250mM and DMSO 0.01% (EnD), heat shock 28°C (28°C) and no treatment (Con) for 6 hours. Samples analysed every 1 hour for 6 hours representative photos shown in rows from left to right (1h, 2h, 3h, 4h, 5h and 6h). Representative of 2 independent experiments with 20 worms**

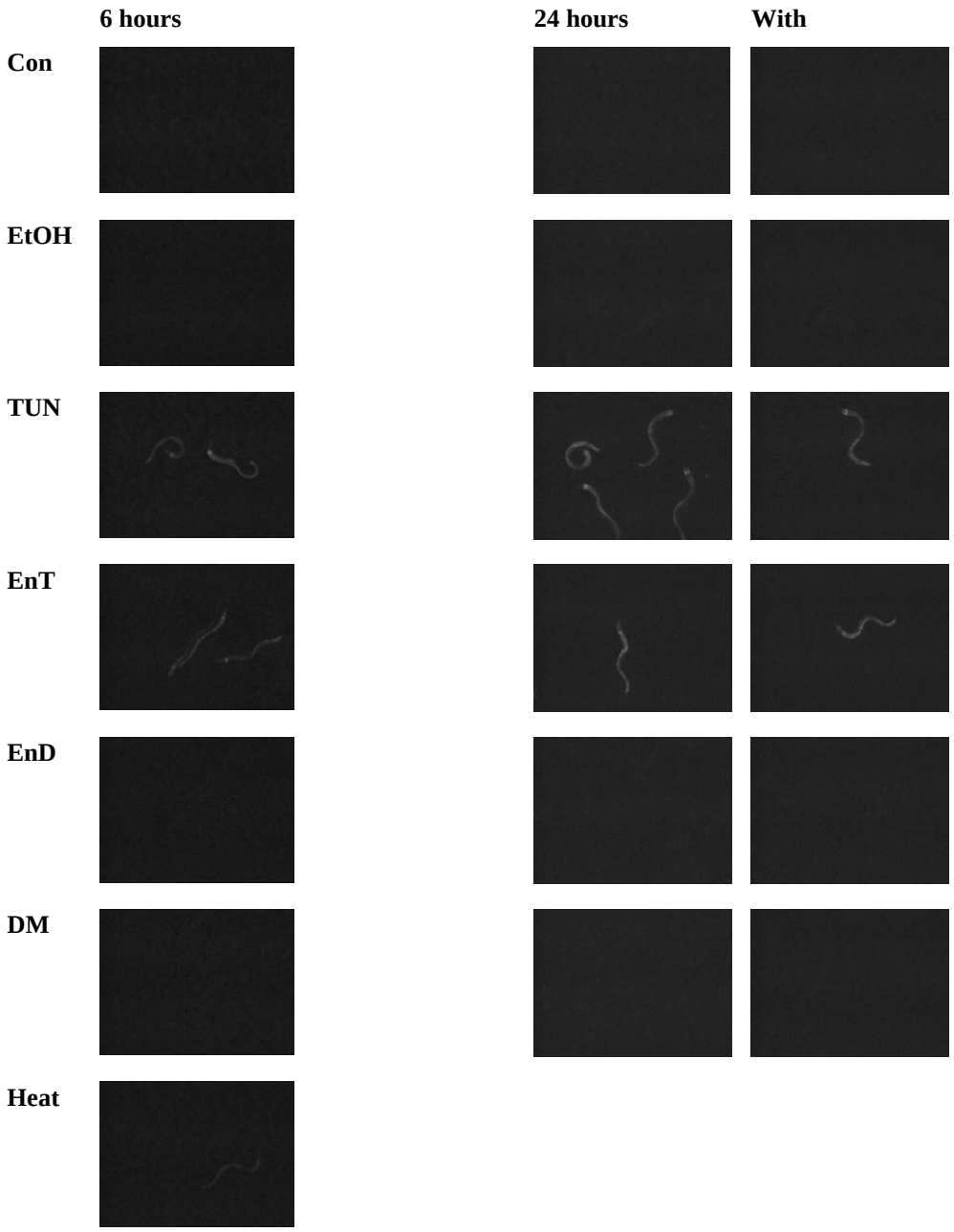


Figure 4.8. *zdIs4* (*phsp4::GFP*) worms conditioned with ethanol and dual tunicamycin treatment for 24 hours and 1 hour post treatment withdrawal.
***zdIs4* (*phsp4::GFP*) worms photographed under 484 nm fluorescent light at 35x magnification. Worms treated with ethanol 250mM (EtOH), tunicamycin 5 µg ml^{-1} (Tun), DMSO 0.01% (DMSO), ethanol 250mM and tunicamycin 5 µg ml^{-1} (EnT), ethanol 250mM and DMSO 0.01% (EnD), heat shock 28°C (28°C) and no treatment (Con) for 24 hours (24) and withdrawal, 1 hour after 24 hour treatment (With).**

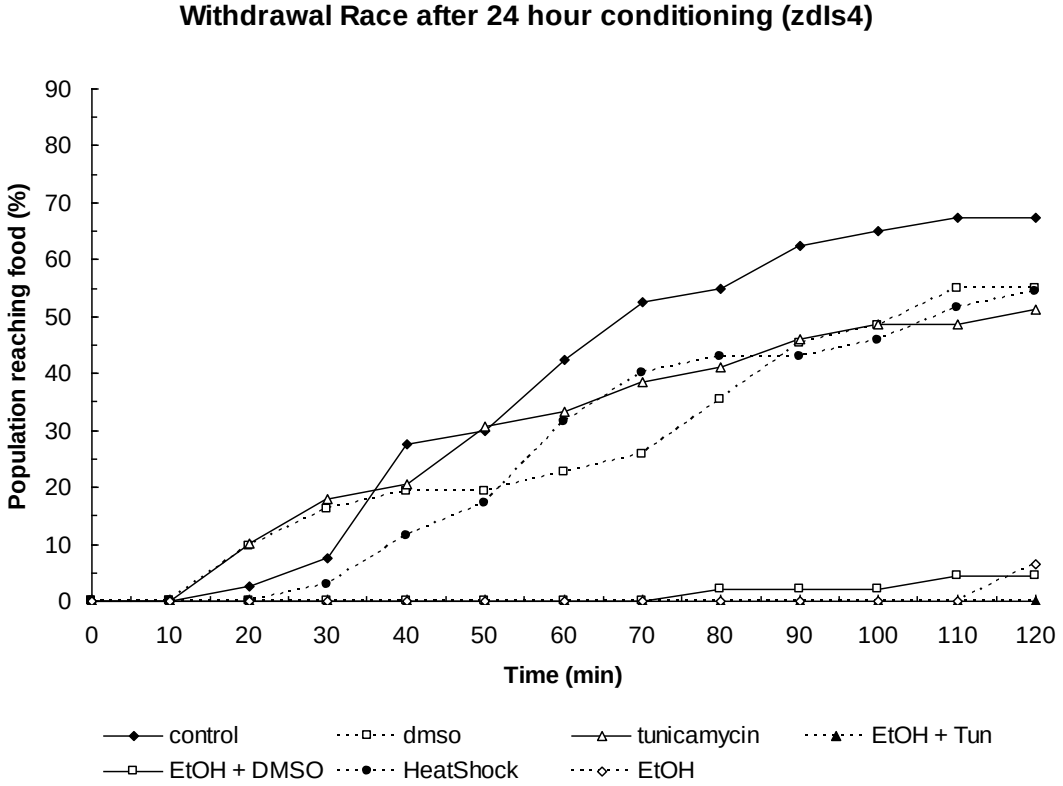


Figure 4.9. Food race of zdIs4 worms after 24 hours ethanol conditioning.
***zdIs4* (*phsp-4::GFP*) worms raced over 5 cm towards a food reward. Population reaching food is recorded every 10 min for 2 hours. Worms conditioned for 24 hours and withdrawn from treatment of: ethanol 250mM (EtOH), tunicamycin 5 µg ml^{-1} (tunicamycin), DMSO 0.01% (dmso), ethanol 250mM and tunicamycin 5 µg ml^{-1} (EtOH + Tun), ethanol 250mM and DMSO 0.01% (EtOH + DMSO), 6 hours heat shock 28°C (HeatShock) and no treatment (control). 68% of the control worm population made the food reward after 2 hours. 51% tunicamycin, 54% DMSO and 54% heat shock treated worm populations made the food. Ethanol treated worms had 7% of the population reach the food. Ethanol and DMSO treated worms had 4% of the population reach the food and ethanol and tunicamycin treatment had no worms make the food reward after 2 hours.**

4.3.4 Investigating the quenching of GFP by ethanol

The results from the *zdIs4* (*phsp-4::GFP*) suggest that ethanol does not play a role in triggering the unfolded protein response in *C. elegans* in behaviourally relevant treatments. It also plays no role in potentiating an UPR response to tunicamycin treatment. These results may have been due to a role of ethanol in changing the fluorescence of GFP through altered protein conformation or misfolding, masking a positive result. Previous literature has highlighted problems using a GFP reporter construct similar to the *zdIs4* strains and suggested that ethanol may interfere with the protein. To investigate the likelihood of false negative results the fluorescence of *pmyo2::GFP* tagged *C. elegans* were compared under ethanol and non-ethanol conditions. Worm strains used were neuronal rescue *slo-1* mutants in the reporter background. *slo-1* should not alter GFP fluorescence, and ethanol has not been reported to alter expression of the GFP construct array. The *pmyo2::GFP* array is expressed solely in the pharynx of *C. elegans*.

larval stage 4 + 1 day worms were treated with M9 saline buffer control, 240mM ethanol or iso-propanol for 5 min after transfer from 5 min incubation in M9 saline buffer. Iso-propanol controls were included as an indicator of solvent effect on fluorescence. Samples were photographed before and after transfer to detect obvious changes in GFP fluorescence (Fig 4.10). The experiment was repeated twice under different cameras, hence the change in colour. No obvious change in fluorescence can be detected between the samples in any of the three treatment conditions.

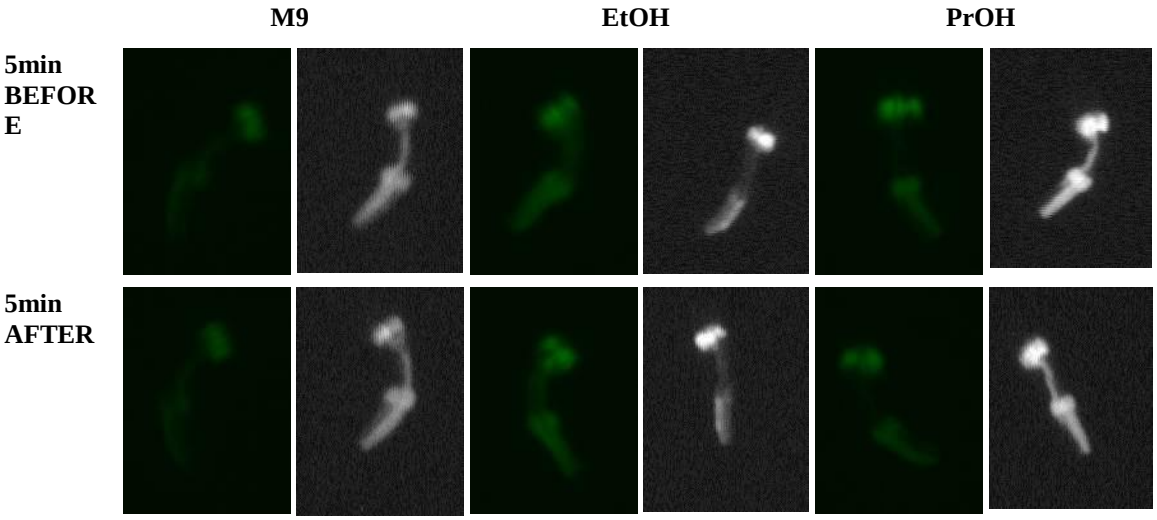


Figure 4.10. Visual comparison of GFP expressing worms before and after ethanol and iso-propanol treatment.
***pmyo2::GFP* worms photographed under 484 nm fluorescent light at 35x magnification. Two sets of worm groups were treated with either M9 control (M9), 240mM ethanol (EtOH) or iso-propanol (PrOH) for 5 min after 5 min incubation in M9 buffer. Worms after 5 min M9 (BEFORE) and 5 min treatment (AFTER) photographed. No difference is observed in fluorescence on control, ethanol or iso-propanol treatment. Repeated experiments differ in camera type only.**

4.3.4.1 *In vitro* GFP quenching by ethanol

An investigation into the changing fluorescence of eGFP in vitro on increasing ethanol concentrations was carried out by the team at Mill Hill (unpublished, Personal correspondence). Increasing concentrations of ethanol made by serial dilution were mixed 1:1 with eGFP and sampled three times. Samples were read from a black-walled, clear bottomed 96 well plate and read with a Tecan-Sapphire fluorescence plate reader (Fig 4.11).

Initial ethanol concentrations reduce the fluorescence of eGFP by approximately 10-20% until recovery at ~250mM. Fluorescence then increases progressively by approximately 70% to 100% on ethanol concentrations up to 2.8M. The difference between scan results is likely due to photo bleaching between scan runs. The 1st scan was done initially at 15:56, whilst the other scans were made subsequent to this at 16:47, 16:49 and 16:50 respectively.

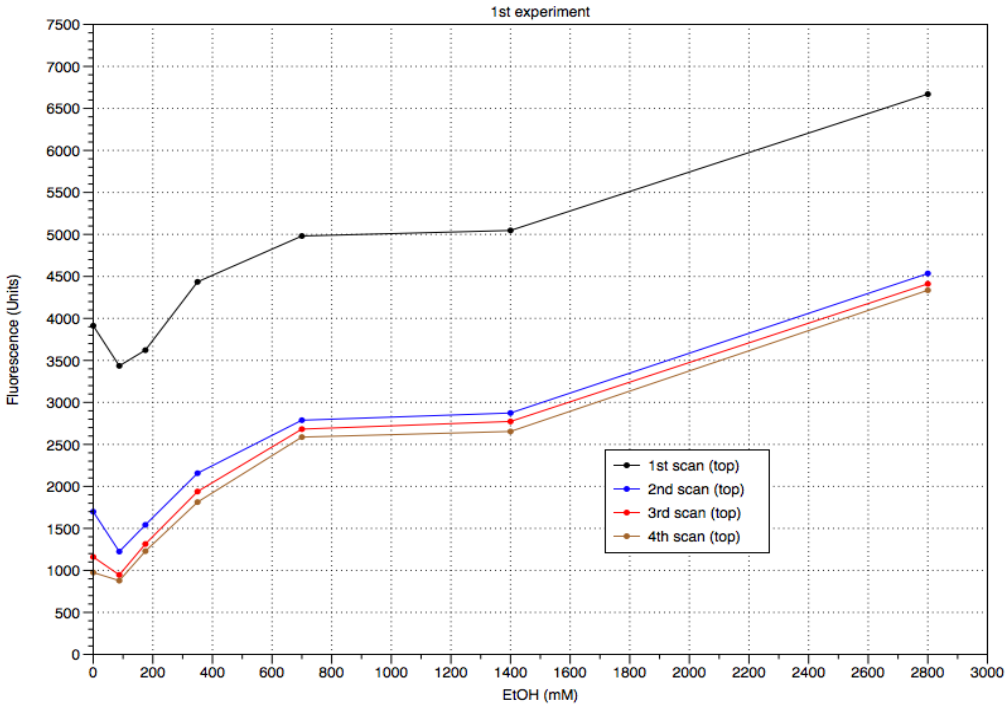


Figure 4.11. Fluorescence of eGFP on increasing concentrations of ethanol as read by a Tecan-Sapphire fluorescence plate reader.
Each point is the mean of three samples. Ethanol concentrations below 250mM cause a slight reduction on fluorescence by 10-20%, with a progressive increase in fluorescence of ~70-100% up to 2.8M ethanol. Difference between scans is likely photo bleaching between scan run times. Ethanol alone gives no fluorescence under the same settings.

4.3.5 Behavioural responses to ethanol in *hsp-4*$^{-/-}$ worms

Initial investigation shows that *hsp-4* is not induced on ethanol treatment and it is unlikely that the UPR mediates ethanol withdrawn behavioural phenotype. To confirm the role of the UPR marker HSP4 in ethanol mediated behaviour gk514 worms, which possess a mutant inactive copy of the *hsp-4* gene, were investigated for effect on ethanol response phenotypes. Initial results suggest that *hsp-4* mutants do not show any difference in ethanol mediated behaviour when compared to N2 wildtype worms (Figure 4.12).

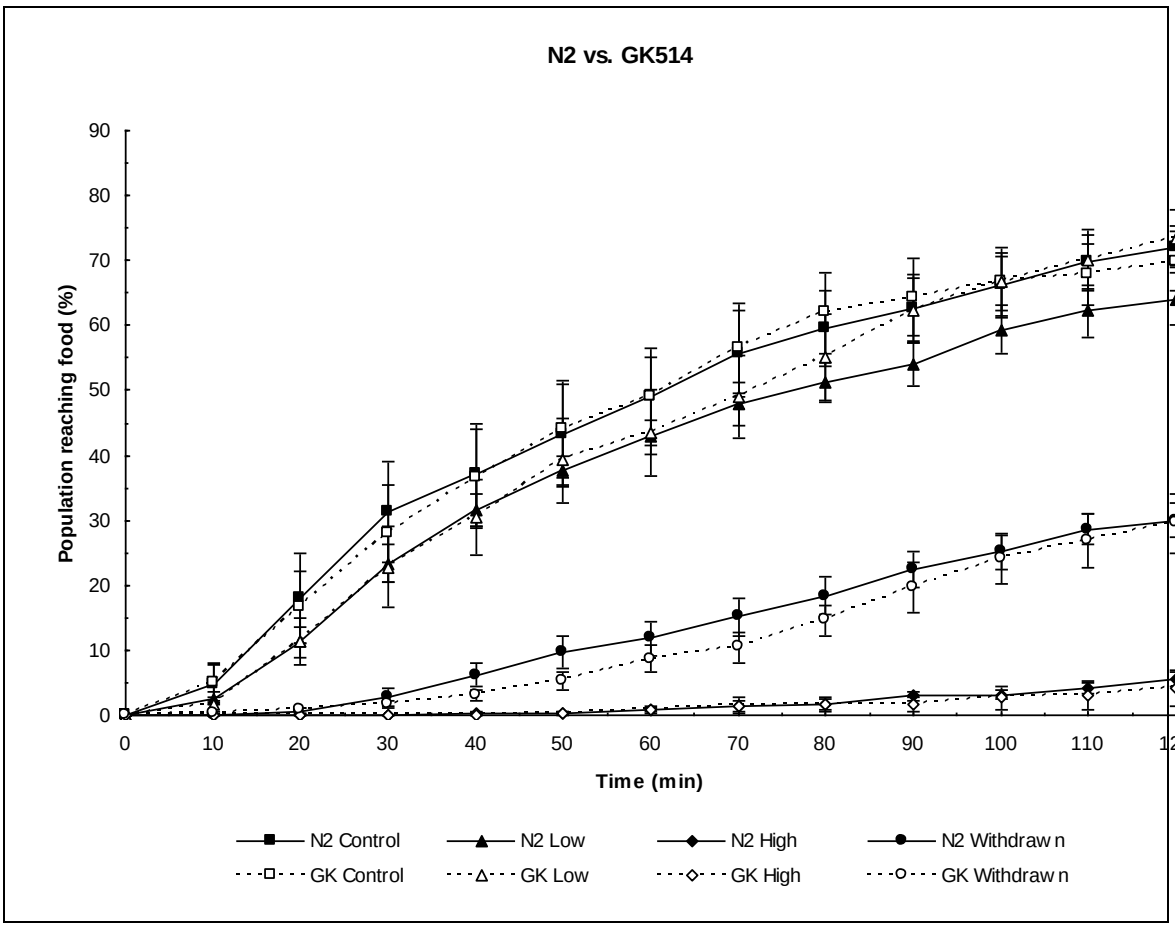


Figure 4.12. Food race of gk514 and N2 worms on 50mM ethanol, 300mM ethanol and ethanol withdrawal.
gk514 hsp-4 $^{-/-}$ **and *N2* worms raced over 5 cm towards a food reward. Population reaching food is recorded every 10 min for 2 hours. gk514 (GK) and N2 (N2) worms treated with either ethanol 50mM (Low), 300mM ethanol (High), or conditioned for 6 hours prior to race with 300mM ethanol and raced on no ethanol (Withdrawn) or with no treatment (Control). Each point is the mean of 6 repeats ±SE.**

4.4 Discussion

The unfolded protein response is an adaptive mechanism for coping with cellular stress. Its wide acting, plastic response is implicated in the pathology of several diseases. The implication of whether the UPR is a consequence or a cause of disease is still to be elucidated. It has been implicated in the underlying problems associated with chronic ethanol consumption, particularly in hepatic steatosis and the development of fatty liver disease. The down regulation of methionine synthase by ethanol is believed to be a trigger for a condition known as hyperhomocysteinemia. Hyperhomocysteinemia has been shown to be a trigger for the unfolded protein response and can lead to a number of conditions including atherosclerosis. Whilst the link between chronic ethanol use and the UPR is being forged, little is known about whether the unfolded protein response is an initial cellular response to ethanol stress. Whilst several components of the UPR have been shown to be transcriptionally upregulated on ethanol treatment, a direct link between acute ethanol response and the UPR is still contentious. The model organism *C. elegans* has been used to dissect the roles of both ethanol treatment and

separately the UPR. It has been shown that treatment with ethanol at 7%, enough to cause complete paralysis of the worm and LD_{50} on withdrawal, can trigger the UPR. More subtle, behavioural phenotypes can be observed on ethanol treatment in the worm at lower concentrations such as intoxication, withdrawal, relief from withdrawal and tolerance. The molecular mechanism underpinning these behaviours is of importance in both medical and neurological research. If ethanol stress can trigger an unfolded protein response in *C. elegans* then the molecular mechanism may underpin aspects of these the behavioural phenotypes. Much of this is likely to arise through homeostatic plasticity which in turn involves molecular remodelling of the nervous system. In several models of synaptic plasticity the processing of neuronal receptors is important and events at the level of the ER appear salient. This reinforces the potential for the ER stress to underpin functional and dysfunctional plasticity associated with adaptive states including those that arise in response to ethanol and other addictive drugs.

4.4.1 Ethanol treatment fails to elicit an *hsp4::GFP* response in *C. elegans*

Evidence from treatment of *C. elegans* suggests that the unfolded protein response is not triggered by treatment with ethanol at behaviourally relevant concentrations. Treatment with ethanol at 450mM for 24 hours failed to induce an *hsp-4* promoter driven GFP positive response when compared to controls. Withdrawal from ethanol also did not induce a *phsp-4::GFP* response. Withdrawal after 24 hours still elicited a behavioural response as measured by the food race assay. Only 17% of ethanol withdrawn worms made it to a food reward after 2 hours in comparison to 87% and 81% of the population in negative controls. Interestingly worms treated with an insult of tunicamycin at 30 µg ml^{-1}, which did trigger an unfolded protein response, also produced a behavioural response. Only 46% of tunicamycin withdrawn worms made it to the food reward after 2 hours, a larger number than the ethanol withdrawn worms. The deficit in locomotion of the tunicamycin withdrawn worms may be a result of the unfolded protein response, which was still activated post treatment. Ethanol withdrawn worms which did not induce an UPR, showed an inability to commute towards the food reward to a greater degree than tunicamycin withdrawn worms. This suggests that the UPR does not play a role in ethanol withdrawal mediated behaviour.

Ethanol does not potentiate a stress response to tunicamycin treatment. Worms treated with 450mM ethanol and tunicamycin 5 µg ml^{-1} show no increased stress response at 6 and 24 hours when compared to separate tunicamycin 5 µg ml^{-1} treatment. If ethanol induced a stress response that was at a level too low to detect with the *phsp-4::GFP* array or worked in a stress pathway independent of *hsp-4* induction, then ethanol treatment might be expected to prime a tunicamycin response. Comparison with tunicamycin 5 µg ml^{-1} control showed no increased fluorescence suggesting that it is unlikely that 450mM ethanol induces a UPR. This is also repeated in the solo 450mM ethanol treatment. Ethanol treatment at higher concentrations for a duration of 24 hours may have induced a small *phsp-4::GFP* response. 1.35M ethanol shows a

slight increase in fluorescence beyond control samples at the 24 hour time point. This ethanol concentration is approaching the reported level used to induce an UPR in *C. elegans* (Kwon et al., 2004; Uccelletti et al., 2004). Caution is noted as the concentrations of ethanol used were not experimentally verified in this experiment.

The unfolded protein response is not triggered over a 6 hour time course on 250mM ethanol treatment and does not potentiate a UPR in tunicamycin stressed worms. Investigating whether an initial early stress response is induced which is missed at the 6 hour time point; treatments were monitored every 1 hour for 6 hours, then again at 24 hours and 1 hour post withdrawal. Ethanol fails to induce a UPR over 24 hours and at early time points.

4.4.2 Ethanol fails to potentiate an *hsp4::GFP* response in *C. elegans*

Adding weight to earlier results 250mM ethanol also fails to potentiate an UPR to tunicamycin induced stress at early, 1-6 h and late, 24 h, time points. Tunicamycin treatment activated the UPR at 4 hours which was similarly seen in ethanol and tunicamycin treatment with no difference in the level of response. Ethanol and ethanol and tunicamycin treatment combined also fail to induce and to prime a stress response on treatment withdrawal. Induction of the UPR may have been tunicamycin specific. 6 hours heat shock at 28°C induced a stress response from 2 hours up until 6 hour point of treatment withdrawal. The UPR failed to persist in the heat shocked worms up to the 24 hour time point. Result indicates that *phsp-4::GFP* response is not tunicamycin specific. Worms withdrawn from treatment were assessed in a food race assay. 68% worms receiving no treatment reached the food reward after 2 hours. Tunicamycin, DMSO and heat shock treated worms had slightly less of the population reaching the food reward at 51%, 54% and 54% respectively. This is in contrast to ethanol treated worms which only 7% of the population reached the food. Ethanol and DMSO combined treated worms had 4% of the population reach the food, similar to ethanol only treatment. Worms treated with ethanol and tunicamycin treatment had no worms make the food reward after 2 hours.

Worms in this assay had lower population numbers make the food reward after the 2 hours than previous race assay. This is likely due to the increased starting population size of the initial assay. Of the 150 worms for each treatment, the mean starting the race was 97 worms, a transfer efficiency of 65%. For the second assay 60 worms averaged a total population size of 40, 66% transfer efficiency. The large numbers of worms increases the worm density at a single spot beginning each race. Worms react by a backwards motion and omega turn in response to body touch. Increasing the worm density at a specific place may increase the time spent in a locomotory response to repel away from the population spot beginning the race. This suggests caution when comparing the two assays. We do see a similar range of phenotypes between the two data sets; with tunicamycin withdrawn worms having a larger population reaching the food

after 2 hours than the ethanol treated worms. Again the UPR is still active in tunicamycin treated worms compared to inactive in ethanol withdrawn worms. Worms treated with both ethanol and tunicamycin combined did not reach the food reward at all. Whilst the result suggests that the use of the two insults is additive, more repeats is needed to confirm that the results differ significantly and are not due to random variation. If ethanol withdrawn behaviour shares a common molecular pathway with tunicamycin withdrawn worms then dual insult would be expected to behaviourally redundant and show the same level of incapacitation as ethanol treatment. If the results are additive however it would suggest a separate molecular pathway underscoring the behavioural phenotypes.

4.4.3 GFP fluorescence is unlikely affected by ethanol presence

The treatment of ethanol on *phsp-4::GFP* worms fails to induce an increase in fluorescence and indicate an unfolded protein response. Treatment of ethanol may induce a response but remain undetected due to quenching of the fluorophore. Using worms that express GFP independently of ethanol treatment it was investigated whether ethanol could cause a change in fluorescence that would lead to a depression of a GFP mediated response. Using *myo2* promoter GFP is expressed solely in the pharynx of worms. A change in conditions from control buffer to ethanol treatment would reveal if ethanol had any quenching effect on GFP in vivo. Include was an iso-propanol control to counter for any change in solution properties which may affect fluorescence. Worms treated with 240mM ethanol did not show any obvious difference in fluorescence output. This suggests that ethanol does not play a role in GFP quenching and that the results from UPR assays are not the result of false negatives. The quenching of GFP was also confirmed using in vitro GFP fluorescence. Using a fluorescent plate reader on GFP increasing concentrations of ethanol were tested for the effect on fluorescence. Initially ethanol decreases GFP slightly up to 100mM which increases to normal levels on 200mM ethanol. The results picked up by the UPR assays are unlikely to be quenched by ethanol and help confirm that the results are not false negatives. Beyond 200mM GFP fluorescence increases on treatment with molar concentrations of ethanol. This suggests that the increase in fluorescence that was seen on worms treated with 1.35M ethanol may be a false positive. Quantification of all samples is needed to determine whether the link between in vitro and in vivo ethanol on GFP is constant.

Investigating the link between the UPR and ethanol mediated behaviour was also looked at the role of HSP-4 on ethanol intoxication. Preliminary data also suggests that the UPR does not play a role in ethanol mediated behavioural responses. Worm race assays on *gk514 hsp-4*$^{-/-}$ shows no difference in response to low levels of ethanol than that of matched paired controls. Work is currently ongoing to elucidate the role that the UPR sensor *hsp-4* plays in ethanol mediated behaviour.

The investigation has shown that the induction of a stress response by ethanol is unlikely and that the UPR does not underlie the behavioural phenotypes seen on ethanol treatment. Ethanol fails to induce a *phsp-4::GFP* response at 250mM and 450mM when conditioned for 24 hours and on withdrawal. Ethanol also fails to potentiate a stress response induced by tunicamycin treatment under the same conditions. The lack of an UPR is likely due to ethanol not triggering ER stress rather than quenching of the reporter GFP. Ethanol withdrawal fails to induce an UPR and exhibits a behaviour resulting in locomotory deficit in food race paradigm. Tunicamycin sustains an UPR on treatment withdrawal and exhibits a less pronounced depression in locomotory function. Preliminary results from *gk514 hsp-4*$^{-/-}$ show no difference in behavioural phenotype on low ethanol doses. These results suggest that the UPR is unlikely to regulate behavioural responses seen on ethanol treatment.

The use of the *pshp-4::GFP* to monitor UPR induction is a widely used and validated reporter system. The results from the UPR assays show clear positive responses from the tunicamycin treated control samples. The negative controls also show a clear stable level of basal GFP with definitive contrast to an induced response. Whilst clear that ethanol does not induce a response to a similar degree as tunicamycin, and that this is not specific to pharmacological insult but to ER stress, samples need to be quantified in a defined region of interest for fluorescence intensity. This would help confirm that ethanol plays no role in triggering an UPR. It can also confirm the link between GFP fluorescence in vitro and in vivo on ethanol treatment. If the in vitro samples show an increase in fluorescence in ethanol conditions that show an in vivo positive response, this may be attributed to ethanol's effect on the fluorophore, rather than an activation of an UPR.

Whilst the *phsp-4::GFP* array is integrated into all cells the UPR response does show increased reporter activity in the endothelial cells of the gut. Endothelial cells are responsible for producing high numbers of secretory proteins and are predominantly affected on ER stress. Whilst able to monitor global UPR induction, an indication of cell type specific UPR induction, such as in neurons, is limited. This is partially covered by investigating the behavioural phenotype of UPR component mutants, in which all cells have a mutant copy.

C. elegans possess a paralog of *hsp-4* with 98% identity, *hsp-3*. HSP3 is believed to play a redundant role in sensing the UPR but diverges in its role in development (Sasagawa et al., 2007). It is unclear how the presence of *hsp-3* affects the reporter construct and the ability to sense low levels of ER stress. As both of the proteins converge through ER sensors and transcription factors to up regulate *hsp-4* it is unlikely that the reporting of the UPR is compromised. HSP3 may pose problems in trying to use *gk514 hsp-4* $^{-/-}$ worms as its function is likely to be redundant. Double mutants are reported to be embryonic lethal and are essential for development. Over expression of *hsp-4* and RNAi of *hsp-3* in an *hsp-4* background may provide a better insight into the role HSP4 plays in ethanol behaviours.

The results of these experiments suggest that ethanol is not acting as an ER stressor directly or with tunicamycin treatment. Previous literature has reported that transcriptional upregulation of UPR elements is observed under ethanol treatment and that chronic ethanol use in mammals triggers the UPR with pathological outcomes. Ethanol concentrations that are lethal to 50% of *C .elegans* in experiments have been shown to trigger the UPR. Whilst these findings suggest that such a high concentration may trigger an UPR, they also may be affected by ethanol on the level of fluorescence detected. This needs to be further validated in vivo before extrapolating to other work.

Work in cell lines suggests that whilst UPR markers might be transcriptionally upregulated this does not translate to a proteomic response on acute ethanol exposure. Higher concentrations of ethanol and chronic ethanol treatment have shown to induce a protein based UPR response suggesting that the UPR may lie in the extremes of ethanol conditioning. Using *C. elegans* we failed to see a HSP4 driven UPR on ethanol treatment which causes behavioural responses. It may be that whilst ethanol may up regulate UPR transcripts, this is not seen at the translational level. If this is replicated in *C. elegans* it may represent a novel mechanism for translational control or a mechanism of action for ethanol. eIF2α controls general translation repression and selective translation of UPR transcripts. It is tempting to hypothesise that ethanol may modulate activation of eIF2α.

4.5 Future work

The results presented here although clear need further evaluation to confirm beyond doubt. Levels of fluorescence need to be quantified and evaluated to ensure a high level of certainty that ethanol does not trigger a low level *phsp-4::GFP* response. The link between high concentrations of ethanol that have been reported in the literature and an increase in eGFP fluorescence on increasing ethanol concentration in vitro could also be verified. RNAi knockdown of *hsp-3* in a *gk514 hsp-4*$^{-/-}$ background and its role in ethanol mediated behaviour could also confirm the lack of an UPR role in behavioural phenotypes. This could also be investigated with dual tunicamycin ethanol treatment withdrawal on locomotion. An additive depression on locomotion would suggest separate pathways whilst a redundant response would point to a common, downstream pathway. The *xbp-1/ire-1* pathways have been shown to control around 80% of UPR transcripts including *hsp-4* induction. Investigation of the behavioural effects of ethanol on this pathway would also be good confirmation of a role for the UPR.

Several reports have highlighted a number of transcripts upregulated by ethanol that corresponds to an induction of the UPR. Evidence that the UPR is not activated at the transcription level by ethanol could further validate this work. If ethanol does modulate UPR

mediated transcription then a proteomic analysis would shed light on why the response is not seen in *phsp-4::GFP* worms and may indicate potential sites of action for ethanol.

5 Discussion

Ethanol mediated behaviour can be modelled in the organism *C. elegans*. The behavioural phenotypes displayed by the worms reflect the conditioning paradigm, ethanol concentration and exposure time; simply paralleling the behaviours of other organisms. Although not directly analogous to the complex behaviours of human-ethanol interaction, *C. elegans* demonstrates the basic depression with increased exposure time or dosage treatment, and subsequent improvement on treatment cessation. Other more subtle behaviours of relief from withdrawal and tolerance have been shown elsewhere. It is possible too that the effects of a lower concentration of ethanol treatment have been touched upon by this study, although results warrant further investigation.

It is clear from this study that relatively high concentrations of ethanol are enough to depress activity in both locomotory output and pharyngeal pumping. This depression of activity is limited to 300mM ethanol treatment with an exposure time of 6 hours. Lower concentrations of ethanol fail to elicit a clear response in either locomotory or pharyngeal measures. Worms show a clear recovery from ethanol exposure as measured by locomotion, showing an increased but not fully recovered ability for a population to taxi towards a food stimulus. The withdrawal behaviour is, in part, governed by a change in bend type and turn frequency, shown elsewhere. This behaviour is more efficient at allowing worms to chemotax than the depressed, shallow movements of the 300mM ethanol intoxicated worms.

Although the effects are pronounced with higher concentrations of ethanol, the picture is far less clear with decreasing ethanol concentration. Investigating how ethanol concentration effects pharyngeal pumping, an interesting observation was noted. The worm's initial reaction to the presence of the food stimulus is affected by the presence of ethanol. Worms exposed to ethanol will move from a food rich area to a food sparse area. Experiments were adjusted to control for the presence or absence of food; as there is a higher pharyngeal pumping rate when food is present and movement off food may have affected results. Depression of pharyngeal activity on exposure to 300mM ethanol remains independent of food availability, as does the lack of effect at lower ethanol concentrations (<300mM). On both food present and food absent test environments, higher ethanol concentrations depress pharyngeal pumping, whereas lower ethanol concentrations have no significant affect.

Environment preference does change with ethanol concentration, with worms exposed to increasing concentrations of ethanol moving from food-present to food-absent areas. Lower concentrations of ethanol, down to 25mM, are still enough to stimulate this behaviour. Either a secondary interaction of ethanol with the environment is being sensed by the worms or a direct behavioural change as a result of ethanol is causing this phenotype. Preliminary experiments

controlling for bacteria-ethanol interaction point to a possible effect on the worms rather than a change in the food environment. Heat-killed bacterial food, and bacteria transferred from exposed and non-exposed environments suggest that there is little change in the bacteria that is sensed by the worms on ethanol exposure. More work is needed to unravel the complexity of this phenotype.

Whilst the worm race assay provides a foundation for studying the distinct 'states' that arise with worm intoxication and conditioning, it is unable to pick up more subtle phenotypes that arise outside the spectrum of gross movement changes that perturb locomotion. The 2 hour experimental time fails to account for changes within that spectrum and initial changes that occur on ethanol intoxication. The race assay provides a useful tool for characterising the mechanisms behind locomotory and chemotaxis behaviour affected by ethanol, but is unable to provide a platform for identifying behavioural changes on lower ethanol intoxication. The pharyngeal pumping rate offers a new angle to explore that can move away from movement and co-ordination behaviour. It also has the advantage of being able to explore more direct measurements of a simplified, yet still complex, neuronal network at the cellular level and relate this back to pharyngeal behaviour. It is unfortunate that initial experiments failed to show any correlation between behavioural change and sub 300mM ethanol treatments, although experiments off food hint at future possibilities.

Whilst pharyngeal pumping was unable to show a distinct clear phenotype on low ethanol intoxication, it did lead to the observation of environmental dispersal in ethanol treated worms. This observation was able to show worms respond to an ethanol environment by either increased movement, at least initially, or an aversion/attraction behaviour. The role of the bacterial lawn is unclear, but initial experiments at least suggest that dispersal is a response to the ethanol. The mechanism behind this phenotype may help understand mechanisms of low dose ethanol intoxication.

All of the phenotypes studied in *C.elegans* provide a useful and descriptive insight in to worm-ethanol interaction. It is through the use of these techniques that the basic mechanisms of ethanol interaction can be dissected to shed light on the molecular basis of behaviour.

5.1 Bioinformatic identification of genes involved in ethanol response

Given the number of studies that show the many ways in which ethanol interacts to control behaviour, and yet the specificities of a distinct pathway still elude researchers after many years of study, it is unsurprising that the database of these molecules is enormous and eclectic. The current collection of information harvested from papers between 2006 and 2009 on 5 organisms contains over 3500 entries on 900 biologically important proteins and molecules. As it stands, the database is a work in progress; the information still needs to be analysed and mined for

specific trends and correlates. There is also scope for the addition of more information and data from other projects. As it stands there is no clear conclusive evidence of one particular pathway contributing to ethanol mediated behaviour, but without proper analysis the question is still open.

The difficulty in managing an information source of this size in part comes from two important facets; when mining data from one source to another the transition of data without adding or losing information, but manipulating the data in such a way that it can be easily used as a resource. The other is the process of manually finding and integrating the data out of the original sources, the slowest and rate determining step in the construction of the database. The first was controlled by the use of an ontological vocabulary. The second by due diligence.

An ontology is a system of controlled terms each with a predefined meaning that may have a hierarchical structure of parent and child terms each with a broader or more specific scope of meaning respectively. By controlling the vocabulary to scientifically defined terms and structures there was an automatic control over how words were interpreted and more importantly loss of data fidelity. The use of a hierarchical vocabulary also has the added bonus of aiding future manipulation and mining of data.

The current database will need to be properly analysed for any possible evidence of linkage between ethanol treatment and behavioural outcomes. One of the difficulties that arise from this task is the interpretation of the data fields. Each field within the database is a line of evidence specifically from a referenced and recorded paper. Whilst this evidence may be data that the paper has presented first hand, it may be a reference of other work in the field. Papers tend to reference similar papers to their field of study so many of the entries within the database will be redundant lines of evidence referencing the same experiments. There must, when analysing trends, be some consideration given to reported bias. A simple choice would be to weigh the evidence fields with respect to the number of papers cited in the original document, in order to counterbalance this problem.

While the database provides an excellent source of material to direct future experiments, it requires a substantial effort to complete and refine to ensure clear evidence can be sourced from it to pursue and support future hypothesis.

5.2 The unfolded protein response and ethanol

The induction of a stress response by ethanol in *Caenorhabditis elegans* is unlikely to result in a UPR that underlies the behavioural phenotypes seen. A *phsp-4::GFP* response used as a marker to indicate unfolded protein stress pathways did not show any evidence of induction on ethanol treatment. This response was absent over a range of ethanol concentrations and treatment time points, as well as on treatment withdrawal. Worms treated with ethanol for a protein stress

response behaved as expected when challenged with a food race paradigm; confirming that whilst the UPR is not elicited by ethanol, the behavioural response is still present.

Worms treated with tunicamycin, a chemical that disrupts protein folding, did respond positively showing an induction of the UPR marker *phsp-4::GFP*. Worms co-treated with both tunicamycin and ethanol failed to induce a response beyond that of tunicamycin alone, suggesting that ethanol is unlikely to potentiate a protein stress response. To ensure that the *phsp-4::GFP* marker responds to other initiators of protein stress, worms were also heat shocked. Heat shocked worms did show an induction of the *phsp-4::GFP* marker all be it with a quicker response time. To ensure ethanol was not interfering with the *phsp-4::GFP* marker and quenching the fluorescence of the GFP, both *in vivo* and *in vitro* experiments were used to show there is no change in overall fluorescence. GFP tagged worms were treated with photographed before and after ethanol treatment and compared for gross changes in fluorescent output. In addition eGFP fluorescence was measured *in vitro* on increasing ethanol concentrations using a Tecan-Sapphire fluorescent plate reader. Both experiments showed no noticeable change in GFP fluorescence for the range of concentrations used in the protein stress response assays.

The *pshp-4::GFP* assay is a widely used and validated system used to monitor UPR induction. The positive induction of a protein stress response and the basal levels of a response absence are in clear dichotomy; allowing for a definitive contrast between treatment states. It is clear on a whole worm level that ethanol fails to induce a protein stress response, and it is not confounded by concentration time or intensity. Whilst the binary nature of the experiments suggest a lack of a response on ethanol treatment alone, a region of interest (ROI) intensity computation analysis of fluorescence would further support the case for the lack of a potentiated response when co-treating with ethanol and tunicamycin. An analysis on specific cell types could also help detail a clearer picture of ethanol response in neuronal tissues, as whole worm responses are dominated by the secretory cells of the gut endothelium which show clearer responses because of their higher protein throughput.

Worms mutant for the UPR response protein HSP-4 showed no significant difference in behavioural response to ethanol. Behaviours consistent with ethanol intoxication and withdrawal in non-mutant worms were observed in the race assays used.While HSP-4 mutant worms show no difference in ethanol mediated behaviour, it is worth noting that *C. elegans* do possess a paralog of *hsp-4* with 98% identity, *hsp-3*. HSP3 is believed to play a redundant role in sensing the UPR but diverges in its role in development. It is unclear how the presence of *hsp-3* affects the reporter construct and the ability to sense low levels of ER stress. As both proteins converge through ER sensors and transcription factors to up regulate *hsp-4* it is unlikely that the *pshp-4::GFP* assay is compromised. HSP3 may pose problems in trying to use *gk514 hsp-4* $^{-/-}$ worms as its function is likely to be redundant. Double mutants are reported to be embryonic lethal and are essential for development. Over expression of *hsp-4* and RNAi of *hsp-*

3 in an *hsp-4* background may provide a better insight into the role HSP4 plays in ethanol behaviours.

Overall it appears that ethanol does not play a role in triggering or potentiating an unfolded protein stress response. Several experiments show that ethanol does not itself trigger a UPR, and an UPR is not needed for ethanol mediated behaviour. In addition the molecular controls for the UPR are not needed for ethanol mediated behaviour.

5.3 Conclusions

C. elegans provides a useful tool for studying the different aspects of ethanol intoxication and ethanol mediated behaviour. It provides a platform in which to understand how the different paradigms of ethanol conditioning influence behaviour and in turn how this behaviour is controlled. This investigation has showed how *C. elegans* can be used to study the different conditioning paradigms of ethanol treatment and that there is still further scope for investigation into the controls underpinning ethanol behaviour.

Documentation of key molecules underpinning the ethanol response from across several species should provide a background for further investigation in *C. elegans*. Whilst incomplete, the vast amount of data collected in the database should provide an essential, complementary lead on behavioural mechanisms controlling both worm and ethanol behaviour. Analysis and further information integration will only add to and aide an essential resource for future studies. Documentation of the limitations of the database should allow for relevant trends to be filtered accordingly and for any future restructuring and cleaning of the data.

Whilst several lines of evidence may point to a possible link between mechanism across species, conservation is not always guaranteed. Resources such as the ethanol database hope to eliminate potential false leads with future analysis. The UPR was a mechanism that had been linked to ethanol treatment, but failed to show any signs of response to ethanol or involvement in ethanol behaviour. The investigation showed no link between protein stress and ethanol treatment. Future database analysis will hopefully lead to similar clear results on both responsive and non-responsive pathways in ethanol and worm behaviour.

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