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FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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An Analysis of Ethanol-Induced Behavioural Plasticity in *Caenorhabditis elegans*

by

Philippa Helen Mitchell

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ABSTRACT

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AN ANALYSIS OF ETHANOL-INDUCED BEHAVIOURAL PLASTICITY IN *CAENORHABDITIS ELEGANS*

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Ethanol is one of the most widely used and socially acceptable drugs in the world. However its chronic use can lead to serious problems including the development of dependence. Alcohol dependence is a chronic, relapsing disorder characterised by tolerance, withdrawal, preoccupation with obtaining alcohol, loss of control over its consumption and impairment in social and occupational functioning. In humans this develops over years, primarily driven by adaptations in many distinct signalling pathways and neural circuits as a result of continued heavy drinking. Whilst alcohol dependence has been extensively studied our understanding of how its distinct targets integrate to produce various behavioural responses remains far from clear.

The nematode worm *Caenorhabditis elegans* is a model genetic organism with a simple nervous system and well-defined behaviour. These nematodes can display plasticity in the form of tolerance to, and withdrawal from, 5-HT or nicotine. They are thus a genetically tractable system in which to investigate the neural substrates of adaptive responses to ethanol. In this simple system the impact of changes at the molecular level on signalling in defined neural circuits and the resultant animal behaviour can be investigated. The aims of this thesis were to establish a *C. elegans* paradigm for alcohol dependence and to use this to define the genetic basis of the ethanol-dependent behaviours of intoxication, tolerance and withdrawal.

Evidence was provided that ethanol equilibrates rapidly across the worm cuticle indicating that the internal concentration closely approximates to the external concentration in which the animal is placed. Ethanol-dependent behaviours were carefully characterised using a variety of behavioural assays. *C. elegans* exhibit distinct behavioural states, corresponding to intoxication and withdrawal, which impair the ability to navigate towards food. Visual and automated analysis defined a sub-behaviour, an increased tendency to form spontaneous deep body bends, which was specifically associated with withdrawal. This was ameliorated by a low dose of alcohol supporting the contention that it arises from ethanol-induced neuroadaptation.

A series of loss of function mutants, were analysed for alterations in ethanol-dependent behaviour. The absence of withdrawal in a strain of worms depleted in neuropeptides (*egl-3*) demonstrated that peptidergic signalling is key to the chronic adaption to, but not to the acute effects of, ethanol. However the neuropeptide receptor NPR-1, previously shown to impact on ethanol responses in *C. elegans,* had no effect on withdrawal behaviour in these assays. Alterations in intoxication and withdrawal behaviour in strains of worms depleted in 5-HT (*tph-1*) and dopamine (*cat-2*) indicated that serotonergic and dopaminergic signalling may also be involved in the ethanol response in *C. elegans*. This study has therefore provided a quantitative analysis of distinct ethanol-induced behavioural states and highlighted a role for neuropeptides and major classes of neuromodulatory transmitters. In particular this data is consistent with the emerging role of neuropeptides in ethanol withdrawal.

DECLARATION OF AUTHORSHIP

I, Philippa Mitchell, declare that the thesis entitled 'An Analysis Of Ethanol-Induced Behavioural Plasticity 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:

- This work was done wholly, or mainly, while in candidature for a research degree at this University;
- No part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as:

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Signed:

Date:

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Abbreviations

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid	**ERK**	Extracellular signal-regulated protein kinase
5-HT	5-hydroxytryptamine/ Serotonin	**GMM**	Gaussian mixture model
A	Absorbance unit	**Glu (E)**	Glutamic acid
ACh	Acetylcholine	**Gln (Q)**	Glutamine
AChE	Acetylcholinesterase	**Gly (G)**	Glycine
ACTH	Adrenocorticotropic hormone	**GIRK channel**	G-protein coupled inwardly rectifying potassium channel
ADH	Alcohol dehydrogenase	**G protein**	Guanine nucleotide binding protein
ANOVA	Analysis of variance	**GTP**	Guanine triphosphate
Arg (R)	Arginine	**EC_{50}**	Half-maximal effective concentration
bp	Base pair	**P rats**	High alcohol preference rats
BLA	Basolateral amygdala	**HPA axis**	Hypothalamic-pituitary-adrenal axis
BNST	Bed nucleus of the stria terminalis	**ISVR**	Institute of Sound and Vibration Research
BEC	Blood ethanol concentration	**IPCs**	Insulin producing cells
BSA	Bovine serum albumin	**ICV**	Intracerebroventricular
C. elegans	*Caenorhabditis elegans*	**ICSS**	Intracranial self- stimulation
CGC	*Caenorhabditis* Genetics Centre	**IP**	Intraperitoneal
CNS	Central nervous system	**kDa**	Kilodalton(s)
CeA	Central nucleus of the amygdala	**BK channel**	Large conductance calcium- and voltage activated potassium channel
cDNA	Complementary deoxyribonucleic acid	**L1**	Larval stage one
CRF	Corticotrophin releasing factor	**L2**	Larval stage two
cAMP	Cyclic adenosine monophosphate	**L3**	Larval stage three
CREB protein	Cyclic adenosine monophosphate response element binding protein	**L4**	Larval stage four
C	Cytidine	**LD50**	Lethal dose for 50% of subjects
d.f.	Degrees of freedom	**lof**	Loss of function
dNTPs	Deoxynucleotide triphosphates	**NP rats**	Low alcohol preference rats
DNA	Deoxyribonucleic acid	**LB**	Luria Bertani (broth/agar)
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders of the American Psychiatric Association	**Lys (K)**	Lysine
DMSO	Dimethyl sulphoxide	**mRNA**	Messenger ribonucleic acid
DA	Dopamine	**Met (M)**	Methionine
ddH_2O	Double distilled water	**NGM**	Nematode Growth Medium
Drosophila	*Drosophila melanogaster*	**NPF**	Neuropeptide F
EPG	Electropharyngeogram recordings	**NPY**	Neuropeptide Y
EGFR	Epidermal growth factor receptor	**NAD/NADH**	Nicotinamide adenine dinucleotide
E. coli	*Escherichia coli*	**NMDA**	N-methyl D-aspartate
EDTA	Ethylenediamine tetraacetic acid	**nAcc**	Nucleus accumbens
EMS	Ethylmethanesulphonate	**OD**	Optical density
EM	Expectation maximisation	**Phe (F)**	Phenylalanine

PCR	Polymerase Chain Reaction
PFC	Prefrontal cortex
P_{open}	Probability of channel opening
PKA	Protein Kinase A
PKC	Protein Kinase C
ROS	Reactive oxygen species
R	Receptor
rpm	Revolutions per minute
RNAi	Ribonucleic acid interference
Sig	Significance
SNPS	Single nucleotide polymorphisms
SDS	Sodium dodecyl sulphate
SSRIs	Specific serotonin reuptake inhibitors
s.e.	Standard error
T	Thymine
TRPC channels	Transient receptor potential canonical channels
TRIS	Tris(hydroxymethyl) aminomethane
TBE	Tris-borate EDTA
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Val (V)	Valine
VTA	Ventral tegmental area
v/v	Volume/volume
w/v	Weight/volume
AMPA	α-amino-3-hydoxy-5-methyl-4-isoxazole propionate
GABA	γ-amino butyric acid

Chapter 1 - Introduction

1.1 Introduction to Alcohol and Alcohol dependence

Ethanol, the only alcohol suitable for drinking, is one of the most widely used and socially acceptable drugs in the world. Alcoholic drinks are widely used in our society to provide disinhibition in social situations and to relieve tension at the end of the day. In the UK, in 2008, 84% of adults had at least one alcoholic drink (Lader, 2009).

However the abuse of alcohol can lead to serious problems. A recent study ranked the harm caused by twenty legal and illegal drugs according to measures of physical harm, social harm and dependence. Alcohol was ranked as the fifth most harmful drug exceeded only by heroin, cocaine, barbiturates and street methadone (Nutt et al., 2007). It was therefore considered to be more harmful than three of the six class A substances assessed.

One of the most damaging effects of alcohol abuse is the development of dependence. Alcohol Dependence is a chronic relapsing disorder characterised by a preoccupation with obtaining alcohol, loss of control over its consumption, tolerance, withdrawal, and impairment in functioning in both social and work related situations (DSM-IV, 1994).

This drives continued abuse of alcohol and can thus lead to damage to the sufferer from medical conditions such as cirrhosis of the liver, heart disease, pancreatitis or Korsakoff's dementia. It can also affect others around them due to factors such as relationship breakdown, absenteeism, violent behaviour or car accidents (Koob and Le Moal, 2006).

Alcohol dependence develops over several years as a result of adaptations in signalling pathways and neural circuits caused by continued heavy drinking. These adaptations cause alterations in behaviour through complex effects in the human brain, leading to further drinking (Koob and Le Moal, 2001). The overall aim of this thesis is to develop and use the nematode *Caenorhabditis elegans,* which has a much simpler nervous system, as a genetically tractable model for some aspects of alcohol dependence in order to facilitate an integrative analysis of this disorder.

This introduction will first describe what is known about the development of alcohol dependence in mammalian systems. Then it will discuss what invertebrate studies can contribute to this field and review the current literature describing the actions of ethanol on invertebrates. It will finally review where *C. elegans* has already been used to model alcohol dependence, which provided the starting point for the investigations in this thesis.

1.2 Reinforcement

Reinforcement occurs when the consequences of an action increase the likelihood of that action occurring again in the future. Positive reinforcement occurs when the action leads to the addition of a sensation perceived as rewarding. The positively reinforcing effect of ethanol drinking is the sensation of euphoria associated with intoxication. Negative reinforcement occurs when the action leads to the removal of an aversive sensation. The negatively reinforcing effect of ethanol drinking is thus relief from ethanol withdrawal (Koob and Le Moal, 2001).

Therefore, whilst ethanol is initially drunk because the effect is pleasurable, as alcohol dependence develops it may increasingly be drunk to alleviate the unpleasant effects of ethanol withdrawal (Koob and Le Moal, 2001).

The reinforcing effects of ethanol intoxication and withdrawal can also be described in terms of an affective state. This can be linked to the activation of reward pathways (Koob and Le Moal, 2006). For example intracranial self- stimulation (ICSS) has been shown to be positively reinforcing in rats. However they will only lever press for ICSS when it is administered above a certain threshold current-intensity. This threshold current-intensity is held to be a measure of the activation of the reward systems of the brain, as, if the reward systems are more activated less additional stimulation will be required to cause a sensation to be perceived as rewarding. Ethanol intoxication has been shown to decrease the threshold intensity at which rats will lever-press for ICSS, and ethanol withdrawal increases it (Schulteis et al., 1995). Thus, during ethanol intoxication rewarding stimuli are perceived as being more rewarding than normal, which is described as a positive affective state, and during ethanol withdrawal rewarding stimuli are perceived as being less rewarding than normal, described as a negative affective state (Koob and Le Moal, 2006).

1.3 The limbic system

The limbic system encompasses those areas of the brain which underlie emotional behaviour (see Figures 1.1 and 1.2). It is the actions of ethanol on these areas that leads to its positively reinforcing and anxiolytic properties, and adaptations in these areas that lead to the anxiogenic and otherwise aversive state of ethanol withdrawal. This is due to the fact that, under normal conditions, emotional processing within the

limbic system can signal the presence of, or prospect for, either reward or punishment in order to guide normal goal-directed behaviour (Purves et al., 2008).

The limbic system is generally considered to include; parts of the orbital and medial prefrontal cortex (PFC), ventral parts of the basal ganglia, the mediodorsal nucleus of the thalamus, the parahippocampal gyrus, the cingulate cortex and the amygdala (Purves et al., 2008). This section will describe the neurocircuitry of those areas relevant to the development of alcohol dependence.

1.3.1 The nucleus accumbens (nAcc)

The nucleus accumbens is a region of the ventral anterior striatum which integrates excitatory inputs from cortical regions (the orbito-medial PFC) and limbic regions, (the amygdala and hippocampus) with dopaminergic inputs from the ventral tegmental area (see Figure 1.1). Projections from the nAcc go to other basal ganglia nuclei which are involved in motor control and these send feedback projections to the PFC. This neurocircuitry indicates that the nAcc is the site of the integration of emotional salience (amygdala), contextual constraints (hippocampus) and executive/motor plans (PFC), with an integrated output that determines the control of goal-directed behaviour (Goto and Grace, 2008).

The nAcc contains a high proportion of GABAergic medium spiny neurons, whose large dendritic trees enable them to integrate a wide variety of inputs. Afferents from the PFC and limbic systems converge onto single medium spiny neurons, indicating that this integration occurs at the level of a single cell (Goto and Grace, 2008).

The nAcc contains two sub-regions, the core and the shell. These have been suggested to have slightly different functions, in that, the core is considered to have a greater role in conditioned responses based on learning, whereas the shell appears to be required for unconditioned reward seeking behaviour (Goto and Grace, 2008).

1.3.2 The ventral tegmental area (VTA)

The ventral tegmental area is a region of the midbrain close to the substantia nigra. It is the site of origin of the mesolimbic dopamine (DA) pathway. Most of the cells in the VTA are dopaminergic projection neurons (77%) but it also contains a high proportion of GABAergic interneurons (16%) (Johnson and North, 1992b;Johnson and North, 1992a). The mesolimbic dopamine pathway, which has been described as the reward pathway of the brain, projects to various structures including the nAcc, the amygdala and the PFC (see Figure 1.1). These DA neurons exhibit transient burst spike firing in response to unexpected rewards or sensory signals predicting reward. By contrast a transient suppression of tonic spike firing is induced by subsequent omission of an expected reward presentation (Schultz, 2002).

Ethanol is one of many drugs that are abused by humans and that may cause dependence. These drugs of abuse come from diverse and apparently opposite classes (central depressants, central stimulants, narcotic analgesic drugs, etc.), suggesting that they act through various different primary mechanisms, as is in fact the case. However use of all drugs of abuse leads to an apparently pleasurable, euphoric effect and can lead to loss of control over drug taking. This is thought to be due to an action common to all drugs of abuse, the activation of the mesolimbic dopamine pathway (Koob et al., 1998). This is considered to drive the main positively reinforcing properties of drugs of abuse.

The burst of spike firing that indicates reward is thought to facilitate the hippocampal drive onto the nAcc neurons promoting the learning of response strategies. Conversely the suppression of tonic firing that indicates the lack of an expected reward is thought to facilitate the cortical drive onto nAcc neurons promoting behavioural flexibility. Thus behaviours that increase dopaminergic firing are reinforced (Goto and Grace, 2008). Many different drugs which are abused by humans due to their pleasurable effects, such as ethanol, cocaine and amphetamines, have been shown to increase DA concentrations in the nAcc (Di Chiara and Imperato, 1988a).

1.3.3 The amygdala

The amygdala is proposed to be involved with the learnt emotional salience of sensory information. It has also been associated with the experience of fear and anxiety, the expression of fearful behaviour (LeDoux, 2003) and with affective behaviour such as depression (Kalia, 2005). It is a complex mass of grey matter close to the hippocampus, consisting of many distinct subnuclei and is richly connected to nearby cortical areas. It can be divided into three major subregions. The medial group has many connections with the olfactory bulb and olfactory cortex. The basolateral group has extensive connections with the cerebral cortex. The central group has connections with the hypothalamus and brainstem. Thus the amygdala receives highly processed sensory information from all the senses, some direct sensory input and input from some more cognitive circuits. Projections to the hypothalamus, brain stem, ventral tegmental area and nucleus accumbens allow it to play an important role in the expression of emotional behaviour (Purves et al., 2008). Alterations in signalling

pathways within the amygdala are proposed to be involved in the anxiogenic effects of ethanol withdrawal (Koob, 2009).

1.3.4 The raphe nuclei

Serotonergic signalling in the brain is also likely to be important in the development of alcohol dependence. The raphe nuclei in the brainstem are the site of projection of the serotonergic neurons of the brain. These innervate many limbic areas including the ventral tegmental area, the nucleus accumbens, the amygdala, the hippocampus, the hypothalamus and the prefrontal cortex (Carlson, 2007).

1.3.5 Summary

Figure 1.1 summarises the neurocircuitry described in this section and Figure 1.2 illustrates its anatomical localisation.

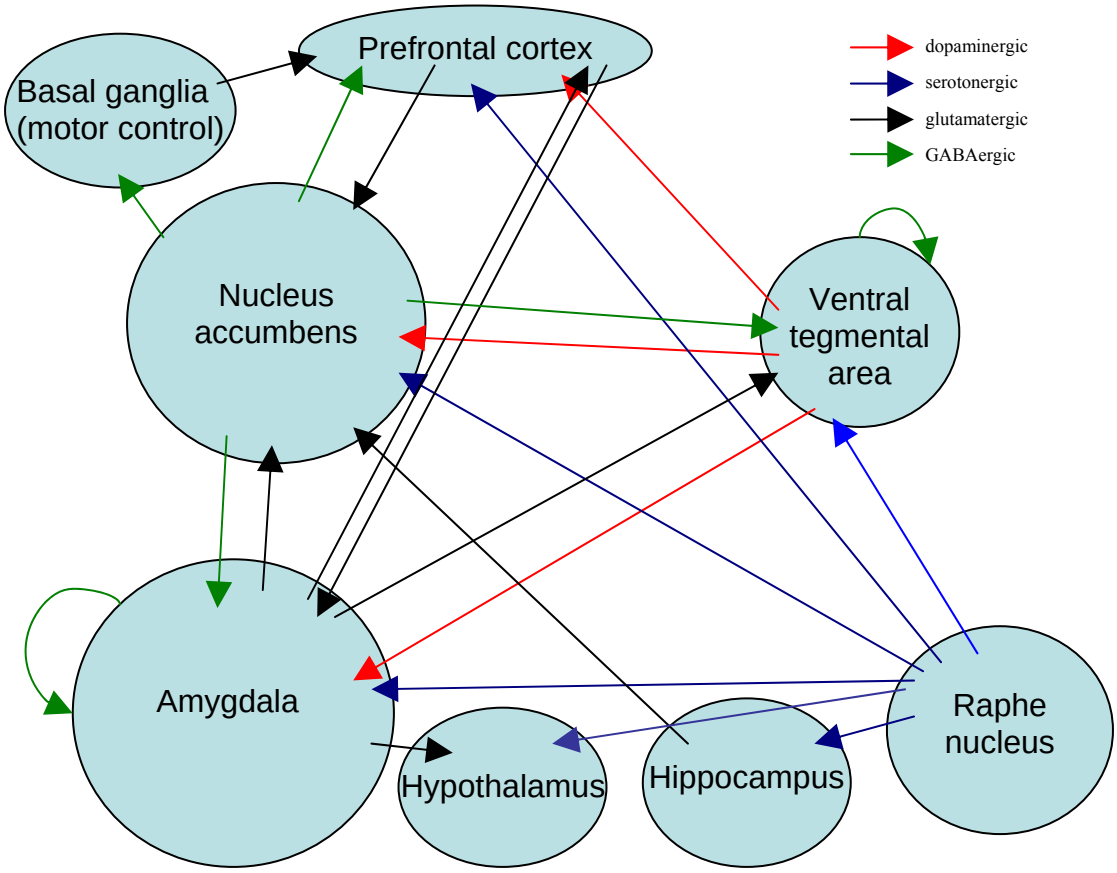


Figure 1.1 Cartoon summary of the neurocircuitry of the areas of the limbic system described in section 1.3 as being relevant to the development of alcohol dependence. (Carlson and Drew, 2006;Purves et al., 2008;Koob and Le Moal, 2006)

Thus in the circuits described above the nucleus accumbens integrates inputs from the amygdala, hippocampus, ventral tegmental area, raphe nucleus and prefrontal cortex to provide integrated outputs that control goal-directed behaviour (Goto and Grace, 2008). Many of the brain regions described above also contain peptidergic receptors and peptide releasing neurons. The location of some of the opioid receptors can be seen in Figure 1.2.

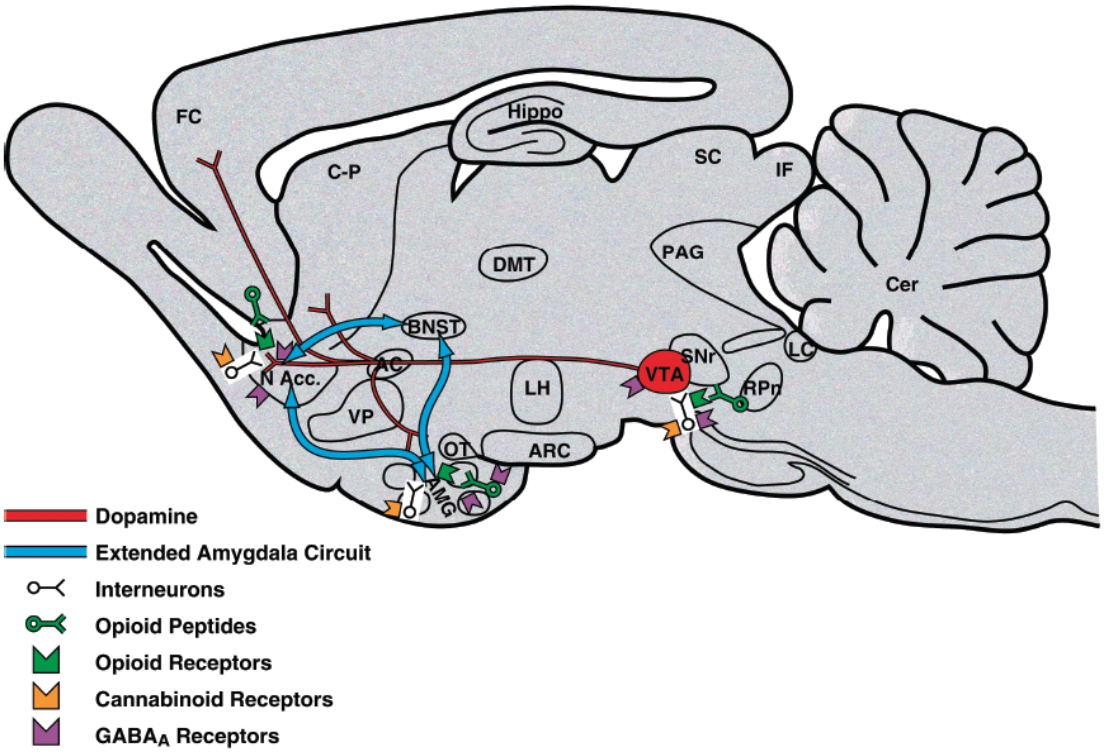


Figure 1.2 Sagittal section through a representative rodent brain illustrating the pathways and brain regions implicated in the acute reinforcing actions of alcohol. AMG, amygdala; BNST, bed nucleus of the stria terminalis; Cer, cerebellum; C-P, caudate-putamen; DMT, dorsomedial thalamus; FC, frontal cortex; Hippo, hippocampus; LH, lateral hypothalamus; NAcc., nucleus accumbens; SNr, substantia nigra pars reticulata; VP, ventral pallidum; VTA, ventral tegmental area. From (Koob and Le Moal, 2006)

1.4 The acute actions of ethanol

The acute effects of ethanol are those which occur immediately on exposure to ethanol, vary with the blood ethanol concentration and continue only whilst ethanol remains in the blood (Koob and Le Moal, 2006). Ethanol is a sedative hypnotic drug which produces behavioural effects such as sedation (decreases in activity) and hypnosis (sleep induction). At lower concentrations (below 20-30mM see Table 1.1) it can produce personality changes and euphoria in humans (Koob and Le Moal, 2006).

The acute behavioural effects of ethanol are fairly well-known in humans, and are summarised in Table 1.1 along with the approximate blood alcohol concentrations that give rise to them. The exact effect of a given concentration of blood alcohol

depends on genetic variation, size, sex and the extent of previous exposure to the drug (Koob and Le Moal, 2006). The table describes the responses to increasing blood ethanol concentrations; in mammalian systems it has been shown that blood and brain ethanol concentrations are identical from ten minutes after intraperitoneal or intragastric administration (Smolen and Smolen, 1989). This gives an indication of the ease with which ethanol, as a small polar molecule, can normally cross membranes and equilibrate.

Blood Ethanol Concentration (BEC)			**Behavioural effects on humans**	
0% (v/v)	0mM	0.0mg/ml	Normal	Normal ↓ Relief from anxiety ↓ Disinhibition ↓ Sedation ↓ Hypnosis ↓ General Anaesthesia ↓ Coma ↓ Death
0.06% (v/v)	11mM	0.5 mg/ml	Personality changes Relief from anxiety Social lubricant (more talkative, assertive, eloquent) Disinhibition	
0.10% (v/v)	17mM	0.8 mg/ml	UK drink-drive limit Significant Disinhibition (life of the party) Impaired judgement Impaired cognition Impaired motor function	
0.19% (v/v)	33mM	1.5 mg/ml	Marked ataxia (staggering, slurred speech) Major motor impairment Impaired reaction time Blackouts (periods of time that cannot be recalled)	
0.38% (v/v)	65mM	3.0 mg/ml	Increased sedation/hypnosis (stuporous but conscious) Approaching general anaesthesia Approaching coma	
0.51% (v/v)	87mM	4.0 mg/ml	Lethal dose for 50% of people	

Table 1.1 Behavioural changes in humans corresponding to increased blood alcohol levels (adapted from (Koob and Le Moal, 2006))

Other mammalian systems have been used as models in which to investigate the acute effects of ethanol. In mice a blood alcohol concentration of 1mg ethanol/ml blood is considered to be the minimum required to produce intoxication (Rhodes et al., 2005), which is similar to the levels required in humans.

Various different behavioural tests can be used to measure different levels of acute intoxication. For example a common measure of extreme intoxication in mice is the loss of righting reflex – the ability of mice to get back on to their feet. After being given a sedating dose of ethanol the blood ethanol concentration at which mice regain the righting reflex is approximately 4-4.5mg/ml in naive mice (Wallace et al., 2006). Thus the sedative-hypnotic effects of ethanol are similar in rodents and in humans..

1.4.1 The mechanism of the biological effects of ethanol

It was initially assumed that the acute effects of ethanol were caused by ethanol partitioning into biological membranes and disrupting their structure. This was due to the fact that ethanol, in common with all volatile anaesthetics fits into the Meyer-Overton plot whereby solubility in olive oil is directly correlated with anaesthetic potency (Kaufman, 1977). However whilst ethanol can decrease the temperature of the gel-to-liquid crystalline phase transition of model membranes, expand membranes and alter the surface charge of membrane lipids, these effects occur with ethanol concentrations in the 500-1500mM range, which would be lethal to humans (Harris and Schroeder, 1981). It is now generally accepted that ethanol acts on protein targets, leading to a wide but selective action on neurotransmitter systems in the brain (Franks and Lieb, 2004). However it is still possible that ethanol is causing a mild disruption in lipid packing in the membrane at concentrations in the 10-100mM range to which certain proteins are particularly sensitive, especially as the majority of ethanol

responsive proteins are membrane bound receptor/ ion channel complexes with multiple subunits in which cooperative interaction between these subunits is essential for function (Avdulov et al., 1994). The interactions between membrane proteins and their lipid environment play important roles in the stability and function of these proteins. These can be specific to individual proteins and include the interactions of aromatic side chains (i.e., Trp, Tyr) with lipids, and interactions of basic side chains (i.e., Lys, Arg) with phosphate groups (Deol et al., 2004).

Ethanol does not have a single target protein, but rather directly interacts with or modifies many different proteins, some of which are summarised below.

Protein target	**Ethanol activates/ inhibits?**	**Ethanol concentration range**	**References**
$GABA_AR$	↑	1-50mM	(Lobo and Harris, 2008;Reynolds and Prasad, 1991)
$5\text{-}HT_{3A}$ R	↑	25-200mM	(Lovinger, 1991;Machu and Harris, 1994)
Nicotinic AChR	↑↓	↑25-100mM α3β4 ↓25-50mM α7	(Narahashi et al., 1999)
Glycine R	↑	10-200mM	(Davies et al., 2004b;Mihic et al., 1997)
GIRK channels	↑	10-200mM	(Kobayashi et al., 1999)
NMDAR	↓	5-50mM	(Lovinger et al., 1989)
P2XR (ATP R)	↑ ($P2X_3R$) ↓(other P2XRs)	5-200mM	(Davies et al., 2005)
BK channels	↑	10-100mM	(Davies et al., 2003;Dopico et al., 1996)
L-type Ca^{2+} channels	↓	50mM-100mM	(Treistman et al., 1991)

Table 1.2 Proteins that have been shown to interact with ethanol at relevant concentrations

The most studied ethanol targets are the $GABA_A$ and glycine receptors. In these it has been shown that two specific amino acid residues in transmembrane domains 2 and 3 are critical for allosteric modulation by alcohols (Mihic et al., 1997) and it has been

suggested that this indicates that these residues form part of an alcohol binding pocket. It has also been shown that mutation of an amino acid residue at the same position in transmembrane domain 2 in the 5-HT_{3A} receptor alters receptor gating and alcohol's modulatory actions. However this study stated that the lack of a relationship between the loss of an enhancing effect of alcohols and any physiochemical property of the substituted amino acids suggested that the changes in alcohol modulation were more likely to be the result of generalised changes in channel conformation rather than specific disruption of an alcohol binding pocket (Hu et al., 2006). Therefore there is still debate about whether alcohol interacts with living systems to exert its acute effects by binding directly to proteins, or by changing the channel kinetics of receptors that contain multiple protein subunits by alterations in their interactions with their lipid environment.

Although the molecular details of ethanol's mechanism of action remain poorly resolved, the behavioural and psychological responses to ethanol have been widely investigated. These broad and complex changes in personality, affective state, cognitive ability and motor reflexes by ethanol support a pivotal role of several of the key mediatory and modulatory pathways of the central nervous system (CNS). These include other signalling pathways than those involving the receptors/channels mentioned above. They may be knock-on effects or as yet undescribed interactions of ethanol.

1.5 Positive reinforcement

1.5.1 Ethanol and the mesolimbic dopamine pathway

Ethanol has been shown to increase the firing rate of ventral tegmental area (VTA) dopamine (DA) neurons *in vitro* (Brodie et al., 1990) and in freely moving rats *in vivo* (Gessa et al., 1985). This has been shown to be at least partly due to a direct action on these neurons, as opposed to a network effect in the VTA (Brodie et al., 1999). Ethanol has also been shown to increase somatodendritic DA release in the VTA (Campbell et al., 1996).

Wistar rats have been shown to self-infuse intoxicating concentrations of ethanol directly into the posterior VTA but not the anterior VTA (Rodd-Henricks et al., 2000), demonstrating that this is a site of action of the positively reinforcing properties of ethanol. This self-infusion behaviour was prevented by co-infusion of an agonist for the inhibitory D_2 autoreceptor, indicating that dopamine neurons in the VTA were required for this positive reinforcement (Rodd et al., 2004b).

This all agrees with the view that ethanol's positively reinforcing effects are mediated through activation of the mesolimbic dopamine system. However the view of the mesolimbic dopamine system as the reward system of the brain has been the subject of debate. Activation of dopamine neurons has been shown in response to a variety of non-rewarding and even aversive events, as long as the event is salient and unexpected, whilst expected rewards do not activate them to the same extent (Horvitz, 2000). It has also been shown that drug 'wanting' (i.e. the motivation to take drugs) is not always directly attributable to the extent of drug 'liking' (i.e. the euphoric effect

of the drug), and it has been suggested that mesolimbic dopamine may be more involved in the former than the latter (Robinson and Berridge, 2001). However it is clear that mesolimbic dopamine is a critical factor in learning motivated and goal-directed behaviour and therefore in positive reinforcement. Some of the mechanisms by which ethanol could be activating dopamine neurons in the mesolimbic pathway will now be discussed.

1.5.2 Serotonergic signalling

The 5-HT_3 receptor is a target of ethanol

The action of ethanol to activate dopamine neurons in the VTA appears to require the ionotropic 5-HT_3 receptor, which can be activated directly by intoxicating concentrations of ethanol. 5-HT can potentiate the ethanol-induced excitation of VTA dopamine (DA) neurons (Brodie et al., 1995). Local administration of a 5-HT_3 agonist increased VTA DA neuron activity and increased DA release in the VTA (Liu et al., 2006). By contrast 5-HT_3 antagonists decreased the number of spontaneously active VTA DA neurons (Rasmussen et al., 1991), and a 5-HT_3 antagonist decreased VTA DA neuron firing and prevented ethanol induced DA release in the VTA (Campbell et al., 1996). In addition co-administration of 5-HT_3 antagonists with ethanol into the VTA completely blocked the acquisition and maintenance of ethanol self-infusion into the posterior VTA (Rodd-Henricks et al., 2003). This suggests that 5-HT_3 receptors in the ventral tegmental area are required for the positively reinforcing effects of ethanol. 5-HT_3 agonists in the nucleus accumbens (nAcc) also increased dopamine release in the nAcc (McBride et al., 2004) indicating a possible additional effect on release from terminals in the nAcc.

G-protein coupled 5-HT receptors may have more minor roles

The action of intraperitoneal (IP) ethanol to increase DA release in the VTA and nAcc is attenuated by 5-HT_{1B} antagonists in the VTA and prolonged by 5-HT_{1B} agonists in the VTA (Yan et al., 2005). 5-HT_{1B} receptors would tend to be decrease firing of neurons. They have been shown to be present on GABAergic feedback projections from the nAcc to the VTA so the increased dopamine release may be partially caused by a reduction of GABA release and consequent disinhibition of the dopamine neurons (Hoplight et al., 2006). However, co-administration into the VTA of a 5-HT_{1B} antagonist did not affect the rate at which rats self-infused ethanol into the VTA, which would argue against 5-HT_{1B} receptors in the VTA being important in ethanol reinforcement (Ding et al., 2009).

5-HT_{2A} agonists have also been shown to potentiate the ethanol induced excitation of VTA DA neurons (Brodie et al., 1995). 5-HT_{2A} antagonists co-infused into the VTA did reduce responding for VTA ethanol infusion (Ding et al., 2009), which indicates that these may be important for reinforcement. Taken together, these studies show that the increased activation of the mesolimbic dopamine system in response to ethanol may be mediated in part by ethanol's actions on 5-HT_3 receptors and modulated by 5-HT_{1B} and 5-HT_{2A} receptors.

5-HT levels are increased in response to ethanol in many brain areas

Acute ethanol increases 5-HT levels in many brain areas such as the nucleus accumbens (Yoshimoto et al., 1992), central nucleus of the amygdala (Yoshimoto et al., 2000), hippocampus (Bare et al., 1998), caudate putamen (Thielen et al., 2001) and frontal cortex (Portas et al., 1994). This increase in extracellular 5-HT in many brain regions does not necessarily mean that its release would be increased in the

VTA as well. One study showed that ethanol decreased firing rates of 5-HT neurons in the dorsal raphe nucleus whilst increasing 5-HT levels in the caudate putamen. This indicates that this rise in 5-HT levels must be a local effect of increased release from 5-HT terminals and/or decreased reuptake (Thielen et al., 2001). This idea is backed up by a study showing that ethanol does inhibit the clearance of 5-HT in the hippocampus (Daws et al., 2006).

The increase in extracellular 5-HT levels in response to ethanol in other brain areas could be important in the motivation for ethanol drinking. Experiments on alcohol-preferring rodents have shown that these have a lower content of 5-HT in the nucleus accumbens (McBride et al., 1995), reduced 5-HT innervation and a higher density of 5-HT_{1A} autoreceptors (Wong et al., 1993). By contrast it has also been shown that specific serotonin reuptake inhibitors (SSRIs) which would tend to increase the availability of 5-HT, decrease ethanol drinking in rodents (Tomkins et al., 2002) and humans (Naranjo et al., 1987). Thus lower 5-HT levels are correlated with higher drinking and vice versa. Reduced levels of 5-HT in areas such as the amygdala would be expected to generate a negative affective state which increased levels of 5-HT produced by ethanol drinking could counteract.

1.5.3 The μ-opioid receptor

Opioid peptides and their receptors are found in various areas of the brain including the ventral tegmental area, the nucleus accumbens, the amygdala and the paraventricular nucleus of the hypothalamus (Koob et al., 1998).

The μ-opioid receptor and its ligand the peptide β-endorphin are also likely to be involved in the rewarding effects of ethanol as shown by various studies. μ-opioid

receptor knockout mice show greatly reduced self administration of ethanol (Roberts et al., 2000). Mice lacking the μ-opioid receptor also showed reduced ethanol consumption and ethanol induced place preference (Hall et al., 2001). μ-opioid receptor antagonists reduced lever-pressing for ethanol in rats that had been previously exposed to ethanol but not made dependent on it (Walker and Koob, 2008).

This rewarding effect is likely mediated through the mesolimbic dopamine (DA) system as it has been shown that a systemic μ-opioid antagonist reduced the increase in dopamine in the nucleus accumbens (nAcc) shell in response to ethanol or sweet food (Tanda and Di Chiara, 1998). This could be due to the fact that it has been shown that opioids acting through μ-receptors hyperpolarise GABAergic interneurons in the VTA leading to the disinhibition of the dopaminergic neurons in the VTA (Johnson and North, 1992b). Ethanol induced excitation of DA neurons is attenuated by μ-opioid antagonists, and μ-opioid agonists do not have a fully additive effect when co-administered with ethanol (Xiao et al., 2007). IP ethanol also increases β-endorphin (the μ-opioid ligand) levels in the nAcc (Marinelli et al., 2004) which is also likely to be positively reinforcing.

1.5.4 GABA

GABAergic signalling is inhibitory and regulates network activity (Stobbs et al., 2004). However the role of GABAergic signalling in ethanol's effects on the dopamine neurons of the VTA is complex and poorly understood (Enoch, 2008). Ventral tegmental area dopamine neurons are under tonic inhibitory control from GABAergic interneurons (Johnson and North, 1992c). There is also GABAergic feedback inhibition from the nucleus accumbens (Neumaier et al., 2002). $GABA_A$

antagonists in the anterior VTA have been shown to attenuate ethanol intake (Nowak et al., 1998), and to reverse the attenuation of ethanol intake caused by a D2 antagonist (Eiler II and June, 2007).

Ethanol however has been shown to enhance GABAergic transmission onto dopamine neurons in the VTA (Theile et al., 2008) which would be likely to inhibit their activity. It has been suggested that this may be a biphasic system whereby ethanol in the VTA activates dopamine neurons directly (see section 1.5.1) and inhibits them indirectly through increasing GABA release. However as described above ethanol could also be acting indirectly to hyperpolarise these GABAergic neurons through an action on μ-opioid or 5-HT_{1B} signalling.

Additionally, injection of a competitive $GABA_A$ receptor antagonist into either the central nucleus of the amygdala (CeA), the nucleus accumbens shell or the bed nucleus of the stria terminalis (BNST) reduced lever pressing for ethanol. These effects occurred at the lowest antagonist dose in the CeA (Hyytia and Koob, 1995). All of these areas contain many GABAergic neurons and have feedback connections to the VTA. Increasing evidence indicates that GABAergic synapses in the amygdala may play an integral role in mediating the acute anxiolytic effects of ethanol (Silberman et al., 2008).

1.5.5 NMDA type glutamate receptors

One study has also demonstrated that GABA neurons in the VTA act in a network whose properties, such as synchronisation, may be governed by NMDA type glutamate receptors (Stobbs et al., 2004). The evidence from this study suggested that ethanol might be acting in the VTA to directly inhibit NMDA type glutamate

receptor-mediated activation of GABA neuronal networks, which might thus activate dopaminergic signalling (Stobbs et al., 2004).

1.5.6 Summary

The positively reinforcing effects of ethanol are therefore considered to be mediated largely through the mesolimbic dopamine system. This is brought about by interactions with many signalling pathways, including serotonergic signalling and opioid peptidergic signalling, which converge on this system.

1.6 Neuroadaptive processes – homeostasis and allostasis

Homeostasis is defined as the process that maintains stability within physiological systems and holds all the parameters of the organism's internal milieu within limits that allow an organism to survive (Koob and Le Moal, 2001). Homeostatic neuroadaptations were first proposed as a cause of drug dependence in 1941 (Himmelsbach, 1941). The concept proposed was that on repeated use adaptations to the drug occur within the CNS, opposing the effects of the drug, thus making it relatively ineffective and leading to drug tolerance. The rapid removal of the drug exposes the state of adaptation, which is removed more slowly than the drug. Because it opposes the effect of the drug, this adaptation causes changes in the opposite direction to those produced by the drug, and these constitute drug withdrawal. Not all adaptations would cause a withdrawal syndrome. Only those that oppose, rather than decrease the action of the drug and remain once the drug has left the system can do this (Littleton and Little, 1994).

Allostasis is defined as maintenance of stability outside the normal homeostatic range, where an organism must vary all the parameters of its physiological systems to match

them appropriately to chronic demands. Allostatic load refers to the cost to the body of being forced to adapt to an adverse or deleterious psychological or physical situation. Drug addiction has been proposed to involve a change in the drug reward set point that reflects an allostatic rather than a homeostatic adaptation. The stability of reward function is maintained by the mobilisation of multiple neurotransmitter and hormonal systems (Koob and Le Moal, 2001). This allostatic adaptation will produce tolerance to the drug in question i.e. the adaptation will cause the drugs acute positively reinforcing effects to be reduced. However removal of the drug will create a state of withdrawal as the drug is now required for stability of reward function.

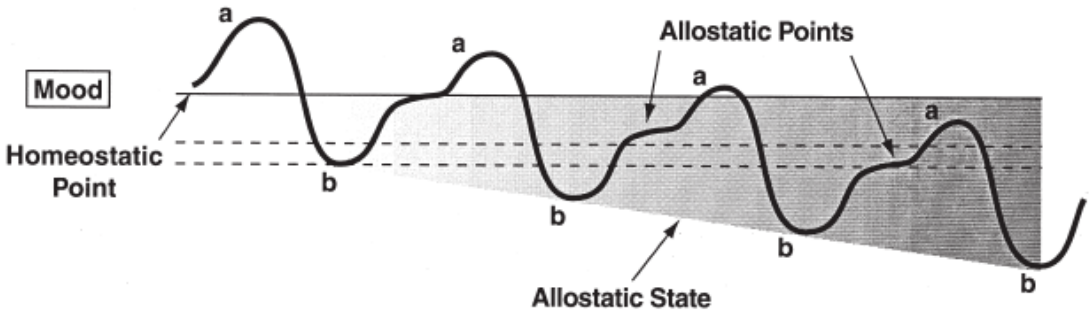


Figure 1.3 The changes in affective state in an individual with frequent drug use that may represent a transition to an allostatic state in the brain reward systems and therefore a transition to addiction. (Koob and Le Moal, 2001). The process marked (a) indicates the increase in a positive mood state in response to the drug, whereas the process marked (b) indicates the increase in a negative mood state due to counter adaptations.

There are therefore two types of adaptations that can occur. Homologous or 'within systems' adaptation involves an adaptation only in the transmitter/ receptor system that has been affected by the drug. Heterologous or 'between systems' adaptation involves adaptations in other systems which counteract the overall effect of the drug. An example of a homologous adaptation would be the increase in binding of the NMDA receptor ligand MK 801 to brain membranes following prolonged ethanol exposure (Grant et al., 1990). As ethanol inhibits the NMDA receptor, an increase in

NMDA receptor levels on brain membranes could be an adaptation that directly opposes this effect.

An example of a homologous adaption that might potentially cause tolerance, but not withdrawal, is the alteration in $GABA_A$ receptor subunit expression seen after long-term exposure to ethanol. It is thought that the composition of GABA receptors in the brain is altered in such a way as to make them more resistant to ethanol without altering the total number of receptors or the extent of normal GABAergic signalling (Littleton and Little, 1994).

An example of a heterologous adaptation is the recruitment of corticotrophin releasing factor (CRF) signalling in the amygdala seen in alcohol dependence. Long term upregulation of CRF1 receptors is observed in the amygdala following a history of dependence and CRF antagonists can reduce emotionality, excessive alcohol drinking and stress-induced reinstatement of alcohol-seeking in post dependent animals (Heilig and Koob, 2007). This is a between systems adaptation that opposes the anxiolytic effect of ethanol, as increased CRF levels in the amygdala are proposed to cause behavioural stress and anxiety responses.

Therefore the development of dependence to a drug is fundamentally dependent on adaptations both within and between brain systems to maintain apparent stability of function in response to the chronic presence of the drug. These adaptations are revealed as a withdrawal state in the absence of the drug.

1.7 Negative reinforcement

As previously mentioned negative reinforcement occurs when an action, such as drinking ethanol, leads to the removal of an aversive stimulus. In alcohol dependence this is seen in 'relief drinking', where alcohol is consumed to relieve the symptoms of alcohol withdrawal. These symptoms include anxiety and a negative affective state. It could also be seen when alcohol is consumed to relieve excessive anxiety caused by other factors such as a genetic predisposition to anxiety or stressful life events. This section will focus on the development of homeostatic and allostatic adaptations that lead to withdrawal over a prolonged period of alcohol use (Koob, 2009).

1.7.1 Clinical withdrawal

Clinical withdrawal in humans is defined as two or more of the following symptoms occurring several hours or up to a few days after someone stops drinking: anxiety, autonomic hyperactivity (i.e., sweating, pulse rate greater than 100), delirium tremens (i.e., anxiety, increased heart rate, sweating, trembling, confusion), difficulty performing tasks involving coordination, grand mal seizures (i.e., convulsions resulting in loss of consciousness and muscle contractions), hallucinations (sights, sounds, or physical sensations on the skin, elevated or decreased temperature), hand tremor, insomnia, nausea, vomiting (DSM-IV, 1994). In rodents withdrawal is characterised by irritability, hyper-responsiveness to stimuli, abnormal motor responses, anxiety-like behaviour, decreased reward sensitivity and seizures (Koob and Le Moal, 2006).

This alcohol withdrawal syndrome has long been characterised as a latent state of hyperexcitability, produced by adaptation to a previously chronically depressed CNS.

It is normally treated with GABA activating drugs such as benzodiazepines. The physical symptoms, such as tremor or nausea, are unlikely to be central to the motivational effects of alcohol withdrawal (Koob and Le Moal, 2006).

1.7.2 Adaptations in the mesolimbic dopamine pathway

As an increase in dopamine in the nucleus accumbens is held to be positively reinforcing, so a decrease in dopamine in the same area is held be aversive, potentially producing a sensation of dysphoria. As mentioned above drugs with aversive properties have been shown to reduce dopamine levels in the nAcc (Di Chiara and Imperato, 1988a). Ethanol withdrawal has been shown to reduce dopaminergic signalling in the mesolimbic pathway although there is some debate as to whether it does this by reducing the number of spontaneously active VTA dopamine neurons (Shen, 2003) or by reducing the firing rate but not the number of active neurons (Diana et al., 1995).

Rats made dependent on ethanol by exposure to an ethanol containing diet as their only source of nutrition for 3-5 weeks showed a progressive decrease in dopamine levels in the nAcc over the eight hours following removal from ethanol, reaching 64% of control levels. When they were subsequently allowed to self administer ethanol, extracellular DA levels in the nAcc were restored to pre-withdrawal levels within ten minutes. Dopamine levels were then maintained at that level by self-administered ethanol for the remainder of the one hour test (Weiss et al., 1996). This was suggested to indicate that the rats regulated their ethanol intake in order to maintain their accumbal dopamine levels at pre-withdrawal levels. This would imply that their drinking was motivated by the negatively reinforcing effect of ethanol to relieve low accumbal dopamine.

It is possible that this reduction in accumbal dopamine could be partially due to direct adaptations of the dopamine neurons, but adaptations in other pathways which act on these neurons have been demonstrated to play a part in this. Some of the main examples of these are described below.

Serotonergic signalling

As described previously acute ethanol causes alterations in serotonergic signalling which are positively reinforcing (see section 1.5.2). Alterations in these signalling pathways in response to chronic ethanol have also been described which contribute to the negatively reinforcing effects of ethanol withdrawal. The action of a 5-HT_3 agonist to increase dopamine release in the nAcc was reduced by a third in rats given eight weeks of free-choice access to ethanol. This effect was shown to persist for at least two weeks after the last ethanol exposure (McBride et al., 2004). The overall levels of 5-HT in the nAcc following eight weeks exposure to ethanol has been shown to be approximately 35% lower than water controls and this effect disappears after two weeks ethanol deprivation (Thielen et al., 2004).

It has been shown that, in rats made dependent to ethanol, ethanol withdrawal causes a progressive reduction in the levels of 5-HT in the nucleus accumbens over an eight hour period. Subsequent self administration of ethanol increased the levels of 5-HT, but not to pre-withdrawal levels (Weiss et al., 1996). Together these studies indicate that adaptation in serotonergic signalling in response to chronic ethanol does occur in the mesolimbic dopamine pathway, and this may therefore be involved in withdrawal.

The κ-opioid receptors and their ligands, dynorphins

κ-opioid signalling is hypothesised to produce a negative affective state. One simple behavioural assay for reinforcing properties is place preference. If a drug is reinforcing an animal will spend more time in places where the drug has been received. κ-opioid agonists produce place aversion and can attenuate ethanol induced place preference, whereas κ-opioid antagonists can act with ethanol to produce place preference at doses of ethanol too low to produce this effect themselves (Matsuzawa et al., 1999). κ-opioid agonists have been shown to decrease dopamine levels in the nAcc (Di Chiara and Imperato, 1988b). This must be an effect on the DA neurons terminals as κ-opioid agonists administered specifically into the VTA did not affect DA levels in the nAcc (Margolis et al., 2006).

There is evidence that κ-opioid signalling is involved in the development of dependence. Animals trained to self-administer ethanol and then exposed to ethanol vapour for a protracted period of time (dependent), subsequently self-administer significantly higher levels of ethanol than control (non-dependent) animals which only received ethanol during the self administration sessions. This is considered to be a model for ethanol dependence. Inhibition of κ-opioid receptor signalling specifically decreased ethanol drinking in rats made dependent in this way but not in non-dependent rats (Walker and Koob, 2008). This indicates that the additional motivation to drink in the dependent rats may be partially caused by increased κ-opioid signalling.

Ethanol withdrawal increases prodynorphin (the κ-opioid ligand precursor) mRNA levels in the nAcc without affecting proenkephalin (a different opioid ligand

precursor) (Przewlocka et al., 1997) and dynorphin concentrations in the nAcc were increased both 30minutes and 21 days into withdrawal (Lindholm et al., 2000). Conversely κ-opioid receptor mRNA levels were reduced following repeated ethanol exposure (Rosin et al., 1999), which was suggested to be an adaptive response to increased dynorphin levels. Another study showed that after repeated ethanol exposure the effect of κ-opioid agonists and antagonists on dopamine levels in the nAcc was altered. κ-opioid antagonists increased dopamine levels more effectively and κ-opioid agonists decreased dopamine levels less effectively (Lindholm et al., 2007). This might again be an adaptive response to increased dynorphin levels in the nAcc.

Overall this indicates that increased dynorphin/ κ-opioid signalling during withdrawal may be involved in the development of a negative affective state involving reduced release of dopamine in the nAcc. This could thus increase the motivation to drink during withdrawal.

There have been fewer studies showing a μ-opioid effect on ethanol withdrawal. However one study showed that ethanol withdrawal decreased the density of μ-opioid receptors in the nAcc (Turchan et al., 1999).

1.7.3 Anxiety, CRF, NPY and the amygdala

One of the major effects of ethanol withdrawal is an increase in anxiety-like behaviours (Baldwin et al., 1991;Knapp et al., 2004;Valdez et al., 2002). The amygdala is implicated in anxiety and fear responses (LeDoux, 2003) (see section 1.3). It has been shown that the amygdala is an important area for ethanol-withdrawal

induced anxiety as 5-HT_{2C} agonists and antagonists affected ethanol-withdrawal induced anxiety, measured by social interaction defects, only when injected into the amygdala and not when injected into either the nucleus accumbens or the paraventricular nucleus of the hypothalamus (Overstreet et al., 2006).

Withdrawal-induced anxiety has been shown to be subject to a 'kindling' process in which it progressively worsens with repeated withdrawals from alcohol. It has also been shown that stressful events can substitute for some of these repeated withdrawals from alcohol, increasing withdrawal-induced anxiety during subsequent withdrawals (Breese et al., 2005).

GABA

Whilst the anxiolytic effects of ethanol are considered to be mediated mainly through alterations in GABAergic signalling (see acute effects of ethanol), the anxiogenic effects of ethanol withdrawal are hypothesized to involve allosteric adaptations in other signalling pathways within the amygdala as well. GABAergic mechanisms within the amygdala are still likely to be relevant as the $GABA_A$ agonist muscimol injected into the amygdala reduced responding for ethanol specifically in rats made dependent on ethanol by continuous vapour exposure as opposed to non-dependent rats, indicating a role for amygdal GABAergic signalling in negative reinforcement (Roberts et al., 1996). However many studies have focused on the recruitment of corticotrophin releasing factor signalling and the inhibition of neuropeptide Y signalling during the development of ethanol dependence. These will now be summarised.

Corticotrophin releasing factor

Corticotrophin-releasing factor (CRF) is a 41 amino acid polypeptide. The highest densities of CRF-positive neurons are found in the paraventricular nucleus of the hypothalamus but there are also CRF positive neurons in the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST) and the brainstem. Hypothalamic CRF acts as the releasing factor for adrenocorticotropic hormone (ACTH) from the pituitary but it can also mediate many other anxiety and stress responses through other regions (Heilig and Koob, 2007).

CRF has two types of receptor known as CRF_1 and CRF_2 receptors, which are both G-protein coupled receptors. Endocrine stress responses are mediated by hypothalamic CRF neurons acting on pituitary CRF_1 receptors. Behavioural stress responses are mediated by extrahypothalamic CRF_1 receptors in the CeA and BNST. CRF_2 receptors act to oppose CRF_1 signalling but their actions are less understood. The CRF_1 signalling pathways that mediate behavioural stress are normally only activated in the presence of uncontrollable stress, indicating that like many neuropeptides CRF is probably only released at high firing frequencies (Heilig and Koob, 2007).

During acute ethanol withdrawal from a two-week ethanol diet, CRF release has been shown to increase in the rat amygdala peaking at 10-12 hours after withdrawal (Merlo et al., 1995). An increase in CRF levels in the BNST has also been shown after ethanol withdrawal in the same paradigm, and in this case it was shown that subsequent re-exposure to ethanol returned CRF levels to normal (Olive et al., 2002).

Withdrawal anxiety, modelled by the time rats spent in the open arms of the elevated plus maze, can be blocked by CRF antagonists in the CeA (Baldwin et al., 1991;Rassnick et al., 1993), indicating that CRF signalling may be mediating the increased anxiety levels seen in withdrawal.

CRF antagonists reduced self administration of ethanol in rats made dependent on ethanol without affecting non-dependent rats (Funk et al., 2007). This effect occurred if the CRF antagonists were administered directly into the CeA, but not if they were administered into the BNST or the nAcc (Funk et al., 2006). As previously mentioned increased CRF signalling in the CeA may mediate the increased anxiety seen in ethanol withdrawal. The increased drinking seen in dependence may thus partially be drinking to relieve this excess anxiety.

An association between alcohol intake and variation at the CRF_1 receptor gene has been demonstrated in humans (Treutlein et al., 2006). This could be a demonstration of non-withdrawal associated negative reinforcement i.e. drinking to self-medicate a genetic predisposition towards anxiety.

The effects of CRF on the hypothalamic-pituitary-adrenal (HPA) axis are also involved in the development of alcohol dependence. Acute alcohol stimulates the release of corticosterone and adrenocorticotropic hormone (ACTH). Chronic exposure led to a dampened neuroendocrine state with reduced corticosterone and ACTH levels. HPA responses to alcohol are reduced in dependent animals and most robust in low-responding non-dependent animals. Decreased expression of CRF mRNA in the paraventricular nucleus of the hypothalamus and reduced sensitivity of the pituitary to

CRF were also seen in chronically exposed animals (Richardson et al., 2008). This dampened neuroendocrine state is associated with a reduced ability to deal with stress. In addition activation of the HPA axis has been shown to be negatively correlated with levels of craving (O'Malley et al., 2002).

Neuropeptide Y (NPY)

Neuropeptide Y is a 36 amino acid peptide. It has four G-protein coupled receptor types Y1, Y2, Y4 and Y5 all of which inhibit the production of cAMP. Centrally administered NPY has been shown to produce an anxiolytic effect, acting primarily through the Y1 and Y5 receptors, in a number of studies (Heilig et al., 1993;Sajdyk et al., 1999).

The central nucleus of the amygdala (CeA) and the basolateral amygdala (BLA) have been shown to be major sites of this anxiolytic effect. Administration of a Y1/Y5 specific agonist into the CeA produces anxiolytic behaviour in the conflict test with a similar potency to intraventricular NPY(Heilig et al., 1993). NPY microinjections into the BLA also produced anxiolytic-like effects in the social interaction test in rats and this was antagonised by a Y1 antagonist (Sajdyk et al., 1999).

It has been hypothesized that NPY and CRF oppose each others actions on anxiety in the amygdala in order to maintain a homeostatic balance, and that this could be mediated by opposing action on the same intracellular signalling pathways (cAMP) (Sajdyk et al., 2004). The cAMP signalling pathway has been implicated in ethanol responses in *Drosophila* (Moore et al., 1998), as described later (see section 1.10).

It has been shown that intracerebroventricular (ICV) administration of NPY does not affect limited access alcohol drinking by Wistar rats (Badia-Elder et al., 2001;Katner et al., 2002). Thus it seems likely that NPY is not involved in the acute effects of ethanol. However under various circumstances which increase ethanol intake, the increase in ethanol intake can be affected by NPY. Neuropeptide Y infused into the CeA abolished elevations in alcohol self-administration in rats made dependent by continuous vapour exposure (Gilpin et al., 2008). Repeated withdrawals from alcohol also lead to an increase in alcohol drinking. This increase can be blunted by intra-amygdala infusion of a viral vector encoding an NPY precursor which will increase NPY levels in the amygdala (Thorsell et al., 2007).

Intracerebroventricular (ICV) administered NPY reduced alcohol intake in rats selectively bred for high alcohol preference (P rats) but didn't affect rats with low alcohol preference (Badia-Elder et al., 2001;Badia-Elder et al., 2003). These P rats also show greater anxiety than NP rats (Pandey et al., 2005). Rats selected as being 'anxious' showed greater preference for ethanol over water than their 'non-anxious' counterparts. In the anxious rats, injection of an NPY antisense viral vector (decreases NPY) into the CeA increased ethanol preference and injection of a NPY precursor viral vector (increases NPY) decreased ethanol preference. Neither injection affected ethanol preference in non-anxious rats (Primeaux et al., 2006). NPY knockout mice show greater anxiety that wild type, greater susceptibility to seizures, much greater ethanol consumption and a resistance to the sedative effects of ethanol (Thiele et al., 1998).

NPY has anxiolytic properties. Ethanol also has anxiolytic properties. One of the symptoms of ethanol withdrawal is increased anxiety (Knapp et al., 2004).
In all of the situations described above ethanol drinking and anxiety are increased and NPY administration can reduce this intake. It is therefore possible that in these situations the increased ethanol drinking is at least partly caused by increased anxiety which is reduced by NPY. This is reinforced by the fact that NPY appears to substitute for the discriminative stimulus properties of ethanol in alcohol preferring P rats (Gilpin et al., 2005).

NPY levels in rats are decreased in the CeA, the medial amygdala and several other brain regions during withdrawal (Roy and Pandey, 2002). In addition NPY levels were decreased in the post-mortem brains of human alcoholics, along with alterations in the levels of many genes involved in the cAMP signalling pathway (Mayfield et al., 2002). It is not known if this reflects an adaptation that occurred in the development of alcoholism or a pre-existing difference which increased the likelihood of the development of alcoholism.

In summary NPY has effects on both anxiety and alcohol drinking and these are both mediated through the amygdala. NPY only appears to decrease alcohol drinking under conditions of dependent drinking, repeated withdrawal, increased anxiety or in rodents bred for high alcohol preference. These are all potentially conditions of increased anxiety. NPY levels may be reduced in withdrawal, contributing to withdrawal-induced anxiety, which is part of the negative affective state that leads to further alcohol drinking. It is also possible that individuals with naturally lower NPY

levels are more susceptible to alcoholism. Thus low NPY may cause part of the aversive state which ethanol can relieve, causing its negatively reinforcing properties.

1.7.4 Summary

The negatively reinforcing effects of ethanol involve the relief of ethanol withdrawal. Ethanol withdrawal may cause increased anxiety due to opposing adaptations in CRF and NPY signalling in the amygdala. Withdrawal may also lead to a dysphoric state due to adaptations in signalling in the mesolimbic dopamine pathway.

1.8 The development of alcohol dependence

The last few sections have described the major factors in the development of alcohol dependence. Initially the acute effects of ethanol are positively reinforcing, meaning that alcohol drinking occurs due to the learnt association with the pleasurable effects of intoxication. However, if ethanol is drunk frequently, over time homeostatic and allostatic adaptations will develop which counter these acute effects. This leads to negative reinforcement, where an aversive withdrawal syndrome develops which motivates relief drinking.

There are several theories describing how alcohol drinking switches from social drinking to compulsive, dependent drinking (Everitt et al., 2008;Robinson and Berridge, 1993;Breese et al., 2005;Koob and Le Moal, 2001). However neural plasticity in response to the continued or repeated presence of the drug is central to all of these theories.

Some of the neuroadaptations that occur have also been described as persisting despite protracted abstinence and contributing to craving and relapse. For example it

has been shown that persistent alterations in CRF signalling can lead to increased sensitivity to stress and increased drinking more than three weeks after removal from ethanol (Valdez et al., 2002;Valdez et al., 2003;Sommer et al., 2008), a time point at which withdrawal anxiety has disappeared. CRF receptor antagonists have also been shown to block reinstatement of ethanol seeking (see section 1.9 below) after footshock stress (Le et al., 2000).

Thus the more complex aspects of alcohol dependence such as relapse, craving and compulsive use can be shown to be underpinned by the homeostatic and allostatic adaptations that occur in response to the continuous or repeated presence of ethanol in the brain.

1.9 Animal models of alcohol dependence

Alcohol dependence is a human disorder. Animal models attempt to parallel various aspects this human condition, but most animal models are limited by the fact that animals do not express the plethora of behaviours that humans produce (Rodd et al., 2004a). For example a model of an alcohol dependent animal relapsing after trying to quit drinking in order to save his job or his marriage has yet to be developed. However a model for relapse after extinction of alcohol seeking has been developed. In this rats are taught to lever press for alcohol (alcohol seeking), the alcohol is then removed so that lever pressing has no result and this behaviour is extinguished. A low priming dose of alcohol, an alcohol related cue or a stressful situation will then reinstate alcohol seeking. These are all factors associated with relapse in humans (Le and Shaham, 2002).

Some other examples of animal models for aspects of alcohol dependence have been already described in this introduction. These include:

- Dependent drinking - Animals trained to self-administer ethanol and then exposed to ethanol vapour for a protracted period of time (dependent), subsequently self-administer significantly higher levels of ethanol than control (non-dependent) animals which only received ethanol during the self administration sessions.
- Conditioned place preference – A preference for places in which intoxication, or another positively reinforcing experience, has occurred.
- Withdrawal anxiety – The increased anxiety seen during withdrawal can be measured by reduced exploration in an open field test, decreased social interaction or reduced time spent in the open arms of an elevated plus maze.

Understanding the mechanisms by which these discrete behaviours occur in animals provides a heuristic framework to understand the development of alcohol dependence in humans.

1.9.1 Contributions from invertebrates

Invertebrate studies allow the investigation of the biological basis of a drug response in an organism with a much simpler nervous system, defined and reproducible behaviours, shorter life cycle and greater ease of maintenance in a lab. Other advantages of using invertebrate model organisms include the numerous molecular biological and genetic techniques that exist for invertebrate experimentation that are not possible with higher eukaryotes. Forward genetic screens enable the unbiased isolation of genes involved in behaviours of interest, and the vast array of mutant strains available assist materially with reverse genetic analysis.

As mentioned above any animal model is limited in that it can only investigate specific aspects of alcohol dependence. Although the more sophisticated behaviours described above do not pertain to their simple nervous systems, invertebrates have been shown to display both ethanol intoxication and tolerance (Wolf and Heberlein, 2003), and can thus be used to study both the acute effects of ethanol and the neuroadaptation which underpins the development of all the more complex aspects of alcohol dependence.

Invertebrate models have many of the same neurotransmitters, receptors and other molecular targets of ethanol as higher organisms. The basis of ethanol's effects can thus be studied from molecules through interacting circuits to behaviour in these simpler organisms, which could go on to inform work in more complex organisms.

This section will discuss what invertebrate models have contributed so far to our understanding of the effects of ethanol, and which aspects of alcohol dependence they could additionally be used to model. The fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* have been the main invertebrate models used to investigate ethanol's effects. However alcohol sensitive potassium channels have also been described in the snail *Lymnaea stagnalis* (Alekseev et al., 1997) and the mollusc *Aplysia californica* (Treistman and Grant, 1990), and a recent paper described a quantitative trait loci mapping experiment for sensitivity to ethanol in the honey bee *Apis mellifera* (Ammons and Hunt, 2008).

1.10 *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* has been increasingly used as a model for ethanol sedation and tolerance over the last decade. *Drosophila* show a response to ethanol similar to that seen both in higher vertebrates and in *C. elegans*, in that in response to ethanol flies initially become hyperactive, then progressively more uncoordinated. With increasing amounts of ethanol they lose their postural control and eventually become sedated (Scholz, 2009).

Many studies of ethanol induced sedation in *Drosophila* have made use of a device called an inebriometer which allows a quantitative assessment of loss of postural control. In this approximately 100 flies are added to a chamber containing many oblique mesh baffles in which ethanol vapour is circulated. After approximately 20 minutes exposure to the ethanol vapour the flies lose the ability to continue standing on the baffles and fall out of the bottom of the chamber. A fraction collector gathers them at three minute intervals and the number of flies in each fraction is counted. Strains of flies which are more sensitive or more resistant to ethanol will have altered elution profiles (Moore et al., 1998).

More detailed analysis of ethanol-induced changes in locomotion has shown that intoxicated files display changes in number of turns, walking speed (Bainton et al., 2000;Singh and Heberlein, 2000) and changes in the frequency and length of activity bouts and time spent moving at different speeds (Wolf et al., 2002).

Drosophila has also been shown to develop tolerance to the sedating effects of ethanol. This has been shown to be caused not by changes in ethanol absorption or

metabolism, but to be 'functional tolerance' based on neuroadaptation to ethanol (Scholz et al., 2000). Two forms of tolerance have been described in *Drosophila*. These are rapid tolerance which is induced by a single short exposure to a high concentration of ethanol, and chronic tolerance, elicited by prolonged exposure to a non-sedating concentration of the drug. Chronic tolerance was shown to require protein synthesis, but this was not the case for rapid tolerance (Berger et al., 2004).

It has also been shown that sensitization can develop to the locomotor activating effects of ethanol (hyperactivity) (Scholz, 2005), and that flies can habituate to the initial startle response to ethanol (Cho et al., 2004). Many studies have used *Drosophila* as a model organism to investigate pathways involved in ethanol intoxication and tolerance. These studies have isolated many mutants with alterations in ethanol induced behaviour. A selection of these studies is described below and they are all summarised in Table 1.3.

1.10.1 Intoxication – sedation

Alterations in both cAMP signalling and EGFR signalling in the insulin producing cells are important for sedation

Alterations in cAMP signalling have been shown to be important in ethanol sedation as loss of functions mutations in *amnesiac*, a neuropeptide which can activate adenyl cyclase, *rutabaga*, an adenyl cyclase and *DCO*, a catalytic subunit of protein kinase A (PKA), have all been shown to increase ethanol sensitivity (Moore et al., 1998), whereas mutants lacking a regulatory subunit of PKA are ethanol resistant (Park et al., 2000).

It was later shown that inhibition of PKA specifically in the insulin producing cells (IPCs) of the dorsal/medial adult brain was sufficient to produce an increased sensitivity to ethanol (Corl et al., 2005). This would be assumed to lead to reduced insulin-like peptide secretion. This study went on to show that reductions in insulin receptor kinase activity, null mutants of an insulin receptor substrate and nervous system specific block of the insulin receptor pathway also produced increased sensitivity to ethanol (Corl et al., 2005). It has therefore been shown that cAMP signalling in the insulin producing cells and its subsequent effects on the insulin signalling pathway appear to be involved in ethanol sedation. The inhibition of PKA in some other brain areas has, in fact, led to ethanol resistance, an indication that cAMP signalling may also have other roles in ethanol sedation (Rodan et al., 2002).

The EGFR/ERK signalling pathway has also recently been shown to be involved in ethanol sedation, as a reduction of function in *happyhour*, a gene which encodes a negative regulator of EGFR signalling, or enhanced EGFR signalling both lead to ethanol resistance. In contrast a reduction in EGFR signalling leads to ethanol sensitivity. In flies with a reduction of function in *happyhour*, but not wild type flies, acute ethanol exposure leads to ERK/Rolled phosphorylation (Corl et al., 2009).

As with cAMP signalling, overexpression of EGFR in the insulin producing cells only, produced ethanol resistance. However, overexpression of EGFR in dopaminergic cells only, also produced ethanol resistance (Corl et al., 2009). In mammalian models, neurons of the mesolimbic dopamine system have been shown to be targets of insulin action (Corl et al., 2005). In *Drosophila,* however, loss of

dopamine signalling has been shown not to affect ethanol sedation, although it does affect ethanol induced hyperactivity (Bainton et al., 2000).

Neuropeptide F is required for normal ethanol sensitivity

Other pathways have also been uncovered which affect ethanol sedation. Neuropeptide F (NPF) is a neuropeptide with homology to mammalian NPY. Flies with either all NPF neurons, or all neurons containing its receptor NPFR, ablated, NPFR RNAi knockdown or temperature sensitive disruption of NPFR function are ethanol resistant. Overexpression of NPF either constitutively or only in NPF neurons increases ethanol sensitivity (Wen et al., 2005). Inhibition of protein kinase C (PKC) specifically in NPF neurons produces ethanol resistance, whereas inhibition of PKA in these neurons has no effect (Chen et al., 2008). NPF signalling is thus important in ethanol sedation.

1.10.2 Intoxication - hyperactivity

Tyramine signalling increases ethanol induced hyperactivity whereas octopamine signalling may be involved in sensitisation

Tyramine and octopamine are hormones, neurotransmitters and neuromodulators in *Drosophila* which are considered to play an equivalent role to adrenaline and noradrenaline respectively. The gene *TβH* encodes tyramine-β-hydroxylase, which is the enzyme required for the synthesis of octopamine from tyramine. *TβH* loss of function mutants have increased tyramine levels and decreased octopamine levels. The gene *inactive* encodes an enzyme required for tyramine biosynthesis. Loss of function mutants of *inactive* show decreased levels of both tyramine and octopamine. *TβH* mutants display increased hyperactivity in response to their first exposure to

ethanol, whereas *inactive* mutants show reduced hyperactivity at their first exposure and increased hyperactivity at their second exposure to ethanol, compared to normal sensitization. This is therefore considered to be an effect of tyramine signalling on ethanol-induced hyperactivity (Scholz, 2005).

1.10.3 Rapid tolerance

Studies have also investigated the development of tolerance to ethanol in *Drosophila.* Chronic tolerance has been shown to require protein synthesis but not octopamine signalling, whereas rapid tolerance required octopamine signalling but not protein synthesis (Berger et al., 2004). More studies have investigated rapid tolerance.

A stress pathway involving the *hangover* gene is required for normal rapid tolerance

A novel zinc finger protein, encoded by the gene *hangover*, was found, in which null mutants displayed reduced rapid ethanol tolerance. This is a separate effect from octopamine signalling as double mutants showed even further decreased tolerance. Heat shock can induce cross tolerance to ethanol. Null mutants in *hangover* showed reduced cross tolerance to heat, an effect not seen in *TβH* null mutants. Null mutants in *hangover* also show reduced ability to tolerate paraquat, the reactive oxygen species (ROS) generating poison, and reduced life-span. Taken together it was concluded that hangover was involved in a cellular stress pathway that is required for normal ethanol tolerance and ROS resistance (Scholz et al., 2005).

Ethanol exposure increases expression of *slowpoke* which is required for rapid tolerance.

The gene *slowpoke* encodes a BK potassium channel. Null mutants in *slowpoke* show no rapid tolerance (Cowmeadow et al., 2005). It has been shown that ethanol exposure increases neurally expressed *slowpoke* mRNA levels and that induction of *slowpoke* was sufficient to produce ethanol resistance (Cowmeadow et al., 2006). Together these data indicate that induction of *slowpoke* during ethanol exposure is required for rapid ethanol tolerance. It is interesting to note that ethanol and benzyl alcohol have been shown to be cross tolerant, and it has been shown that during benzyl alcohol sedation cAMP response element binding (CREB) protein binds to the *slowpoke* promoter region, and that this is required for its sedation-induced upregulation (Wang et al., 2009).

1.10.4 Summary of genes and signalling pathways implicated in the ethanol response in *Drosophila*

Gene/ Signalling pathway	Hyperactivity	Sedation	Rapid tolerance	Reference
PKA/ cAMP	↓	↓ (in insulin producing cells)		(Moore et al., 1998;Park et al., 2000;Corl et al., 2005;Rodan et al., 2002;Wolf et al., 2002)
EGFR		↓ (in insulin producing cells and dopaminergic cells)		(Corl et al., 2009)
Insulin peptides		↓		(Corl et al., 2005)
Dopamine	↓			(Bainton et al., 2000)
$GABA_B$		↑	↓	(Dzitoyeva et al., 2003)
NPF/ NPFR		↑		(Wen et al., 2005)
PKC		↑ (in NPF neurons)		(Chen et al., 2008)
fasciclin II		↓		(Cheng et al., 2001)
Synapsins			↓	(Godenschwege et al., 2004)
Small GTPases	↓	↓		(Rothenfluh et al., 2006)
homer		↓ (in the ellipsoid bodies)	↑	(Urizar et al., 2007)
Tyramine/ octopamine	↑ tyramine (octopamine may affect sensitisation)		↑ octopamine	(Scholz, 2005;Berger et al., 2004)
slowpoke			↑	(Cowmeadow et al., 2005;Cowmeadow et al., 2006)
hangover			↑	(Scholz et al., 2005)
djwa			↑	(Li et al., 2008)

Table 1.3 Genes and signalling pathways involved in the response to ethanol in *Drosophila*.

In conclusion the fruit fly *Drosophila melanogaster* is an invertebrate model which has been used to discover a wide variety of genes and pathways involved in ethanol sensitivity and tolerance. Notably as in mammals, neuropeptide signalling in the form of neuropeptide F and the insulin peptides has been shown to be important in the

ethanol response in *Drosophila* as have the modulatory amine neurotransmitters such as dopamine, tyramine and octopamine.

1.11 *Caenorhabditis elegans*

This study will use *C. elegans* as a model organism to investigate the development of alcohol dependence. *C. elegans* is a small (1mm), free-living nematode worm that is found in the soil or in decomposing organic matter in most temperate regions of the world (Felix, 2007). It was first described as a separate species in 1900 (Maupas, 1900) and in the late 1960's it was selected as a model organism by Sydney Brenner (Brenner, 1974). This was due to its translucent body, simple nervous system, amenability to genetic analysis, and ease of maintenance in a laboratory. It later became the first multi-cellular organism to have its genome entirely sequenced (The *C.elegans* sequencing consortium, 1998).

C. elegans usually reproduces as a self-fertilising hermaphrodite, although males do occur at low frequency. This reproductive system is very convenient for genetic analysis. There are 959 somatic cells in the hermaphrodite and 1031 in the male. Almost every cell in the body develops in the same fashion in every individual of the species with the only exceptions being 11 pairs of cells in which one of each pair will take one fate and one the other (Sulston and Horvitz, 1977). This predictability and simplicity of development and anatomy is very useful for experimentation and has enabled a complete description of the cell lineage of the nematode (Sulston and Horvitz, 1977).

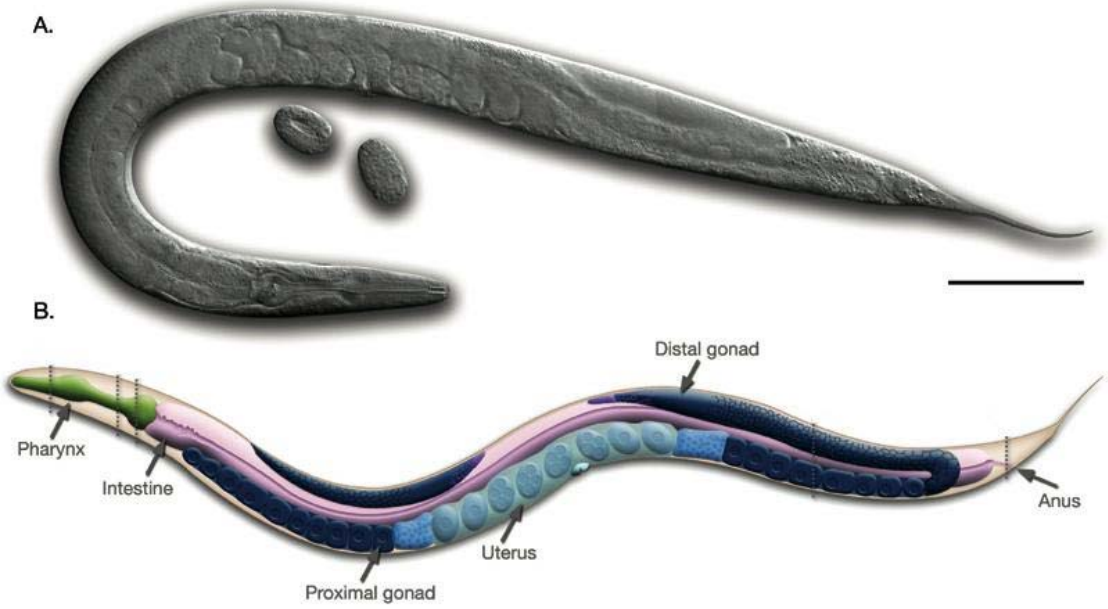


Figure 1.4 The adult hermaphrodite. A. Microscope image of the adult hermaphrodite. Scale bar is 100μm. B. The major anatomical features of the adult hermaphrodite. Adapted from (Altun and Hall, 2006).

Figure 1.4 shows the anatomy of an adult hermaphrodite. The male differs mainly in the tail and the gonad. The body is entirely transparent which, along with the predictability of development, makes it easy to study.

An advantage of working with *C. elegans* is that it has a relatively short life cycle. The life cycle lasts less than three days at 25°C, three and a half days at 20°C or six days at 15°C. There are four larval stages L1-4 between the egg and the adult worm. An additional state is possible during starvation. The L2 larvae may enter the dauer state rather than proceed to L3 in the absence of food. In this state it can survive for many weeks. Encountering food will cause it to continue its development by entering larval stage 4.

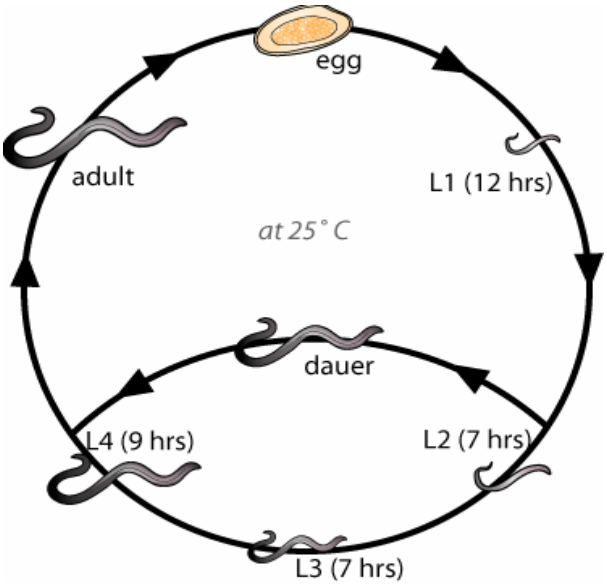


Figure 1.5. Life cycle of *C. elegans* (L1-4 indicates larval stage 1-4)

1.11.1 Behaviour as an analytical tool for *C. elegans*

C. elegans have many well-defined behaviours that can be assayed to provide information about the effect of drugs and/or mutations on the worm. These range from the very simple to the more integrative and adaptive behaviours (de Bono and Maricq, 2005). An example of a simple locomotory behaviour is that *C. elegans* move with a smooth, sinusoidal motion on agar plates. This motion is causes by a wave of successive contractions of the dorsal and ventral longitudinal body wall muscles passing along the worm. This simple behaviour can be measured by counting the frequency of body bends, the shape of body bends or the overall speed (Hart, 2006).

The complete structure of the nervous system is known, having been reconstructed from serial section electron micrographs so that the location of every neuron and its synapses and gap junctions has been determined. There are 302 neurons in the adult hermaphrodite which can be divided into 118 classes (White et al., 1986). These are located in the ventral nerve cord, the pharynx, the tail and the circumpharyngeal nerve

ring. There are 39 classes of sensory neuron, the function of many of which is known due to laser ablation studies, 27 classes of motor neuron and the remainder are interneurons (White et al., 1986). The major neurotransmitter released by many but not all of these neurons is known and these include many of the major neurotransmitters found in vertebrates such as acetylcholine, glutamate, GABA, 5-HT and dopamine (de Bono and Maricq, 2005). In addition many *C. elegans* neurons contain dense core vesicles which are likely to contain neuropeptides and the *C. elegans* genome is predicted to contain 113 neuropeptide genes (Li and Kim, 2008).

This level of understanding has enabled analysis in which the microcircuits controlling specific behaviours can be unravelled. An example of one of these circuits is the locomotory control circuit (see Figure 1.6). This controls forwards and backwards movement in the worm. It contains six classes of motor neuron and five classes of interneuron (called the command interneurons) and controls both forward and backward locomotion. Three classes of motor neurons (DA, DB and DD) innervate the dorsal muscles and three classes (VA, VB and VD) innervate the ventral muscles. Of these, the excitatory, cholinergic DA and VA neurons control backward movement when activated by the AVA, AVD and AVE interneurons, and the also excitatory and cholinergic DB and VB neurons control forward movement when activated by the PVC and AVB interneurons. The DD and VD motor neurons are inhibitory and GABAergic and are activated by the motor neurons that innervate the opposite side to them to provide reciprocal inhibition. They thus enable co-ordinated movement (Chalfie and White, 1988). If a drug is observed to affect locomotion it is therefore altering this simple circuit or one upstream of it in some way.

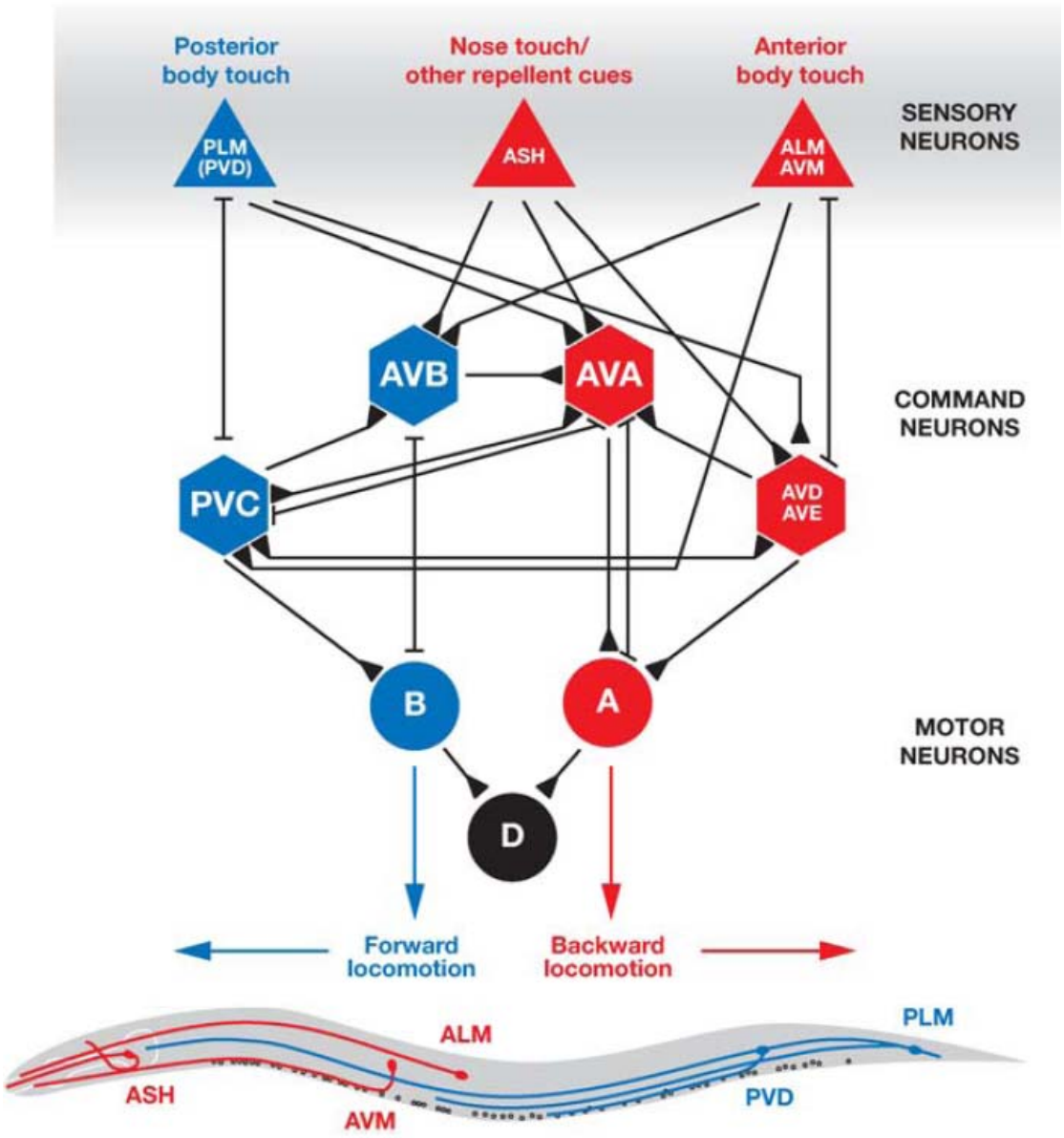


Figure 1.6 The locomotory control circuit. From (de Bono and Maricq, 2005)

The increasing extent to which microcircuits controlling specific behaviours are described means that a careful behavioural analysis can be increasingly used to predict which circuits or even neurons a drug is likely to be acting on. Video imaging has increasingly been used to assist this behavioural analysis as it allows more aspects of behaviour to be examined per assay.

This has enabled a good understanding of a worm's normal behaviour on agar plates to be gained. Worms spent periods of time moving forward (runs), interrupted by periods of time turning (pirouettes) (Pierce-Shimomura et al., 1999). These pirouettes include reversals followed by changes of direction, reversals followed by omega turns and unaccompanied omega turns. An omega turn is a turn of greater than 135° in which the worms head touches or almost touches its tail (see Figure 1.7). Worms can also display reversals not followed by changes of direction. Reversals can be of varying length, with longer reversals being more commonly followed by omega turns (Gray et al., 2005).

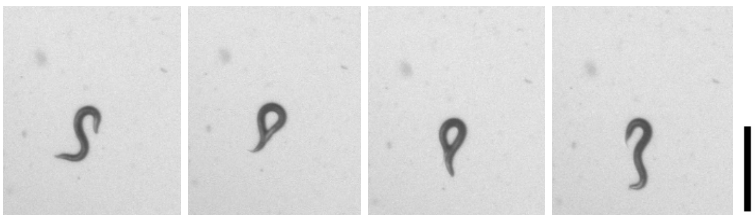


Figure 1.7 Example omega turn. Scale bar represents 1mm.

Alterations in these behaviours in response to different past and present environmental conditions enable *C. elegans* to display more complex integrative behaviours such as chemotaxis towards food, foraging behaviour or altered locomotion when food becomes available. A circuit for navigation during foraging behaviour was described consisting of three main layers of interneurons between the amphid sensory neurons and the command interneurons described above (Gray et al., 2005) (see Figure 1.8). This will be discussed in greater detail in Chapter 5. Briefly three main behaviours were described. Dwelling occurs when a worm is in contact with food. It consists of a worm moving forward slowly, with a high frequency of short reversals followed by low angled turns. This enables the worm to stay in contact with the food. Local search occurs shortly, (5-12 minutes), after a worm has been removed from food. It consists of faster movement interspaced with a high frequency of long reversals and omega

turns and a lower but still reasonably high frequency of short reversals. After the worm has been off food for a longer period (35-40 minutes after removal from food) a plastic response occurs and they enter a dispersal state associated with infrequent reversals and omega turns. The result of this is, that upon food running out the worm first searches the local area thoroughly and then moves further afield (Gray et al., 2005).

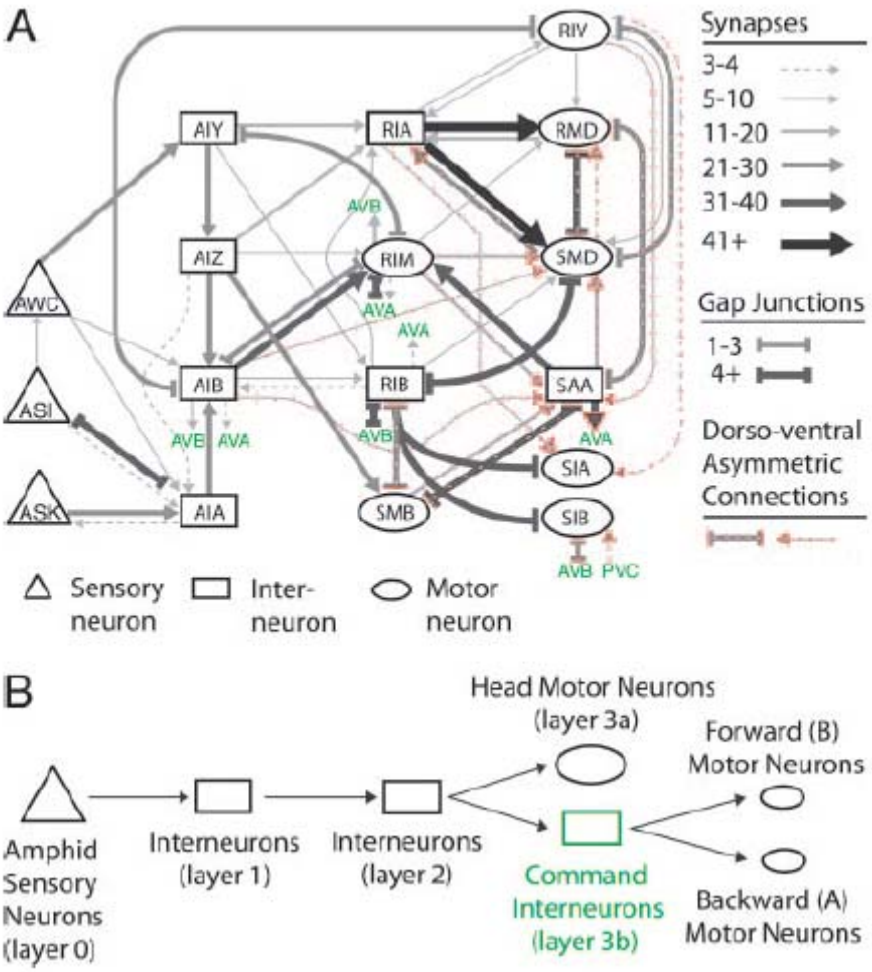


Figure 1.8 A circuit for navigation during foraging behaviour. From (Gray et al., 2005).

C. elegans have also been shown to be capable of longer term plasticity; one example of this is that on a temperature gradient they will move towards a temperature at which they have been previously cultured, or away from one at which they have been

previously starved (Hedgecock and Russell, 1975). They can also habituate to tap stimuli (Rankin, 1991), and become tolerant to the chronic presence of external 5-HT (Schafer and Kenyon, 1995). Thus they can show both very simple easily measurable behaviours and more complex, plastic behaviours which demonstrate their ability to adapt to chronic stimuli and to retain adaptations over a period of time.

1.11.2 Genetic basis of nervous function

Many genes and proteins are conserved between *C. elegans* and humans, including many of those that have been identified as playing a possible role in the effects of ethanol. One example of this is the large conductance Ca^{2+} activated K^{+} (BK) channel, which is found in the human brain and muscle and a homologue of which (SLO-1) has been shown to affect the response to ethanol in *C. elegans* (Davies et al., 2003). In Table 1.4 some of the major signalling pathways that have been described in this introduction as being involved in the development of alcohol dependence are listed. With them are suggested *C. elegans* strains that could be used to investigate their role in the ethanol response in the worm.

Signalling pathway	**Described in section**	***C. elegans* strains**
GABA	1.4 The acute effects of ethanol 1.5.4 Positive reinforcement -GABA 1.6 Neuroadaptive processes – the allostasis theory 1.7.1 Negative reinforcement – Clinical withdrawal 1.11.4 *Drosophila melanogaster* - Summary	*unc-25 e156* A null mutation in an enzyme required for GABA biosynthesis
5-HT	1.4 The acute effects of ethanol 1.5.2 Positive reinforcement - The 5-HT_3 receptor and other 5-HT signalling 1.7.2.1 and 1.7.3.3 Negative reinforcement – Serotonergic signalling	*tph-1 mg280* A null mutation in an enzyme required for 5-HT synthesis
NMDA	1.4 The acute effects of ethanol 1.5.5 Positive reinforcement - Dopamine-glutamate interactions 1.6 Neuroadaptive processes – the allostasis theory	*eat-4 ky5* A null mutation in the glutamate transporter protein
Other glutamate receptors	1.12.1.2 *Caenorhabditis elegans* – Biological basis of intoxication	
Dopamine	1.5.1 Positive reinforcement – The mesolimbic dopamine pathway and others 1.7.2 Negative reinforcement – Adaptations in the mesolimbic dopamine pathway	*cat-2 e1112* A null mutation in an enzyme involved in dopamine synthesis
Neuropeptides – opioid signalling	1.5.3 Positive reinforcement - The μ-opioid receptor 1.7.2.2 Negative reinforcement – The κ-opioid receptors and their ligands, dynorphins	*egl-3 ok979* A null mutation in an enzyme required for peptide precursor processing *npr-1 ky13* A null mutation in the NPR-1 neuropeptide receptor
Neuropeptides – CRF signalling	1.6 Neuroadaptive processes – the allostasis theory 1.7.3.1 Negative reinforcement – Corticotrophin releasing factor	
Neuropeptides – Neuropeptide Y	1.7.3.2 Negative reinforcement – Neuropeptide Y	
Neuropeptides - other	Both insulin peptides and neuropeptide F in 1.11.1 *Drosophila melanogaster* – Intoxication – Sedation The NPR-1 neuropeptide receptor in 1.12.2 and 1.12.3 *Caenorhabditis elegans* – Tolerance and Withdrawal	
The BK channel	1.11.3.2 *Drosophila melanogaster* – Rapid tolerance 1.12.1.2 *Caenorhabditis elegans* – Biological basis of intoxication	*slo-1 js379* A null mutation in the pore forming α subunit of the BK potassium channel

Table 1.4 Major signalling pathways described in this introduction with relevant strains of *C. elegans*. Strain descriptions from http://www.wormbase.org

The level of conservation between *C. elegans* and humans has enabled *C. elegans* to be used as a model to investigate the molecular basis of increasingly complex human diseases. For example, it has recently been employed to study Aβ toxicity in Alzheimer's disease (Wu and Luo, 2005).

Overall *C. elegans* is a powerful system in which to achieve an integrative analysis of the effect of a drug of interest, from the behaviour of the whole organism, through the circuits affecting this behaviour to the proteins on which the drug is acting. For example see Guest et al. (Guest et al., 2007). A thorough understanding of how this can occur in *C. elegans* can go on to inform work in more complex organisms in which the relevant circuits may not be so amenable to investigation.

1.11.3 *C. elegans* as a model for alcohol dependence

Alcohol dependence, as described above, is a chronic, relapsing disorder characterised by tolerance, withdrawal, a preoccupation with obtaining alcohol (craving), a loss of control over its consumption (compulsive use) and impairment in social and occupational functioning. This disorder develops over some years as a result of allostatic adaptations in signalling pathways and neural circuits as a result of continued heavy drinking. In one respect the use of *C. elegans* as a model is limited in that they cannot readily provide insight into the higher cognitive aspects of human addiction such as craving, loss of control over consumption, impairment in social functioning or stress/cue induced relapse.

However *C. elegans* can show plasticity in their behaviours in response to their environment as described above, and they can adapt to the chronic presence of a drug. An example of this is that 5-HT stimulates egg laying, however wild type animals

exposed to 5-HT overnight accumulated unlaid eggs, and were unable to lay eggs in response to a fresh dose of 5-HT (Schafer and Kenyon, 1995). It has also been shown that adapted worms that were transferred to plates without 5-HT exhibited a strong inhibition of egg laying after removal from 5-HT, which was described as a withdrawal effect (Carnell et al., 2005).

This indicates that *C. elegans* are likely to be useful for modelling the alterations in neural signalling pathways which underlie the development of alcohol dependence. These adaptations would result in tolerance to the effects of ethanol and may result in a withdrawal response when ethanol is removed.

In fact a model of nicotine dependent behaviour in *C. elegans* has been described (Feng et al., 2006). In this paper worms that had been incubated overnight with nicotine were shown to have developed tolerance to the locomotion stimulation effect of nicotine and to exhibit a stimulation of locomotion when removed from nicotine, which the authors described as a withdrawal response. They used this model to identify the TRPC (transient receptor potential canonical) channels as being involved in this response. The same group have also investigated the acute response to cocaine in *C. elegans* (Ward et al., 2009).

In addition *C. elegans* have recently been shown to display increased ethanol preference after chronic exposure to ethanol, indicating that adaptations to chronic ethanol in *C. elegans* may cause an increased motivation to obtain ethanol in a similar manner to that seen in higher organisms (Lee et al., 2009).

This study will therefore investigate intoxication in response to acute ethanol and the development of tolerance and withdrawal in response to chronic ethanol in *C. elegans*. The next sections will discuss how these behaviours will be defined and what previous studies have investigated these effects.

1.11.4 Intoxication

Behavioural response to intoxication

The response of *C. elegans* to acute ethanol has been previously investigated in a number of behavioural assays. Acute ethanol has clear effects on locomotory behaviour. Davies et al. showed that *C. elegans* placed on agar containing 100-500mM ethanol had a dose-dependent reduction in the speed of locomotion, the amplitude of body bends and the rate of egg laying (Davies et al., 2003). The response of worms to ethanol over this range of doses was confirmed in a different assay by Graham et al. who demonstrated that in the thrashing assay in liquid *unc-18* loss of function worms with a wild type transgenic rescue of the *unc-18* gene, which were thus assumed to behave in the same way as wild type, showed hyperactivity at 22mM and dose-dependent inhibition of locomotion over the range 200-500mM ethanol (Graham et al., 2008). Kapfhamer et al. performed a dispersal assay on ethanol containing agar plates. This involved worms being placed in the centre of a 10cm plate with food around the edge and the proportion of worms reaching the food being recorded. They showed inhibition at 400mM but not at 200mM ethanol (Kapfhamer et al., 2008). Eckenhoff and Yang described the EC_{50} for the inhibition of *C. elegans* locomotion as 487 +/- 44 mM when scoring worms for movement on a scale which measured the fractions of worms that were either moving normally, moving slowly or

completely still (Eckenhoff and Yang, 1994). All of these experiments point towards an inhibitory effect of ethanol on locomotion at concentrations greater than 100mM.

At concentrations higher than those described above inhibition of locomotion increases leading to eventual paralysis and death. Morgan and Sedensky showed that the EC_{50} for immobility in the worm was 1050mM after 5 minutes in ethanol solution (Morgan and Sedensky, 1995). They determined that in liquid the inhibition of locomotion reached a steady state within five minutes. They also recorded that exposure to ethanol led to an initial hyperactivity followed by a progressive lack of co-ordination followed by immobility and unresponsiveness to tap. This was confirmed by Kwon et al., who also noted that even after up to 6 hour exposures to 1200mM ethanol worms could recover completely within 10 minutes (Kwon et al., 2004).

C. elegans behaviours other than locomotion have been shown to be affected by acute ethanol. Reduced egg laying has been described by several groups (Davies et al., 2003;Kwon et al., 2004) as has a reduction in touch sensitivity (Kwon et al., 2004;Morgan and Sedensky, 1995) and a reduction in pharyngeal pumping (Kwon et al., 2004;Mitchell et al., 2007). It has also been shown that ethanol increased the amplitude of the SLO-1 dependent current in the *C. elegans* CEP mechanosensory neurons at concentrations of 20mM and 100mM in an in vivo patch clamp recording (Davies et al., 2003).

Biological basis of intoxication

Several studies have investigated the biological basis of the response to acute ethanol in *C. elegans*. Genetic screens were performed for mutants resistant to the effects of

ethanol on locomotion and egg-laying at concentrations that strongly inhibit movement but do not cause complete immobility. These isolated 28 mutants with resistance to ethanol of which thirteen were alleles of the gene *slo-1* (Davies and McIntire, 2004;Davies et al., 2003). This encodes the main pore-forming subunit of the BK potassium channel. This study showed that neuronal expression of *slo-1* was required for ethanol sensitivity. It also showed that ethanol activates *C. elegans* SLO-1 in vivo by increasing the frequency of channel opening. This would tend to inhibit the quantal content of synaptic vesicle release, whereas the resistant *slo-1* mutants would have increased vesicle release (Wang et al., 2001).

The BK potassium channel has been implicated in the response to ethanol in both mammalian systems (Dopico et al., 1996) and *Drosophila* (Cowmeadow et al., 2005) as well as *C. elegans*. However intriguingly, whilst the loss of the *slo-1* gene has been described as causing ethanol resistance in *C. elegans*, the loss of the *slowpoke* gene in *Drosophila* has an opposite effect in that it prevented the development of tolerance (see section 1.10.3).

Various proteins more directly involved in synaptic vesicle mediated transmitter release have also been identified which affect the behavioural response to ethanol in *C. elegans*. RAB-3/A is a small G-protein which interacts directly with synaptic vesicles to regulate their release. In null mutants of *rab-3* synaptic vesicle populations at synapses were depleted to 40% of normal levels and synaptic transmission was depressed (Nonet et al., 1997). Worms with null mutations in this gene show significant resistance to the behavioural effects of 400mM ethanol. This was also seen

with loss of function mutants in *aex-3*, which encodes a RAB-3 guanine nucleotide exchange factor (Kapfhamer et al., 2008).

UNC-18 is a syntaxin binding protein. It can bind syntaxin in the closed conformation (Mode 1), which inhibits vesicle fusion, in the open conformation (Mode 2) or when syntaxin is associated with the SNARE complex (Mode 3), which promotes vesicle fusion. A worm containing a version of UNC-18 with a single point mutation that specifically inhibits Mode 3 binding and thus should decrease vesicle release, shows resistance to both the sedative (100-500mM) and the stimulatory (22mM) effects of ethanol (Graham et al., 2008).

It is interesting to notice that whilst all of these mutations confer resistance to ethanol some decrease and some increase synaptic vesicle release, indicating that the mechanism of action is complex.

Other studies looked at the biological basis of immobilisation by much higher concentrations of ethanol. One of the first was by Morgan and Sedensky which identified eight genes that affected sensitivity to immobilisation by ethanol (Morgan and Sedensky, 1995). These are *unc-79*, *unc-1*, *unc-9, fc21, fc20, fc34, fc23* and *fc30.* All of these genes affect the response to at least some anaesthetics as well as ethanol.

The same group have gone on to show that the *fc21* strain, which is hypersensitive to immobilisation by ethanol, encodes a mutation in *gas-1,* which is a 49kDa subunit of complex 1 of the mitochondrial electron transport chain, and that at least one of the

ways that ethanol causes immobility in nematodes is by reducing complex 1 activity (Kayser et al., 2003).

Another group ran a genetic screen for worms resistant to paralysis by 1200mM ethanol. Nine mutant alleles were isolated, many of which could not survive freezing even after extensive outcrossing, indicating that they might be involved in membrane fluidity. One of the genes isolated which could survive freezing, *jud-4*, was shown to encode a novel protein with a limited homology to mammalian Homer proteins (Hong et al., 2008). As mentioned earlier a Homer protein has also been shown to be involved in ethanol sedation in *Drosophila* (see section 1.10.4).

A microarray study analysed genes in *C. elegans* for which expression was altered by 15min, 30min or 6 hour exposures to 1200mM ethanol (Kwon et al., 2004). They identified 230 genes in total that were affected by ethanol, 219 of which were affected by the 6hr exposure. The heat shock protein family genes were the only category of genes in which a significant proportion of genes showed a significant transcriptional increase, they are presumably involved in protection against ethanol toxicity. It is possible there are stress pathways involved in ethanol tolerance in *C. elegans* in a similar manner to that involving the *hangover* protein in *Drosophila* (see section 1.10.3).

Transcription of the gene *glr-2* which encodes a glutamate receptor was increased at 15 minutes and remained at a high level at all time points. Glutamate receptors of various types have previously been implicated in the ethanol response (Krystal et al.,

2003;Sanchis-Segura et al., 2006). However no other glutamate receptors, and no other genes implicated in the ethanol response, were identified as being affected.

On another note Eckenhoff and Yang investigated the effects of pressure on the ethanol response in *C. elegans* (Eckenhoff and Yang, 1994). It is a curious fact that high pressures have been shown to antagonise ethanol intoxication in some organisms. However this did not occur in *C. elegans*, in fact the effects of pressure and ethanol were additive. The authors suggested this was due to the lack of glycine transmission in *C. elegans,* as glycine receptors have been implicated in pressure antagonism of ethanol effects. However it is potentially also possible that this could be an effect of differently structured lipid membranes, as different composition of membranes can be shown to affect the response of human proteins to ethanol (Crowley et al., 2003).

1.11.5 Tolerance

Tolerance is defined as a decrease in the response to a given concentration of ethanol after exposure to ethanol. It is almost invariably present in alcohol dependent individuals but can also be present in many non-alcohol dependent heavy drinkers. It can be separated into tolerance caused by increased liver clearance of ethanol (dipositional tolerance) which can double in dependent patients and tolerance due to adaptation in the CNS (functional tolerance), which plays a much greater part (Koob and Le Moal, 2006). This form of tolerance can be separated into acute 'within session' tolerance or rapid and chronic 'between session' tolerance. Acute tolerance occurs during a single drinking session. Rapid tolerance is seen on the second exposure to ethanol after a single high concentration exposure. Chronic tolerance is an effect of repeated or long term exposure to ethanol.

Tolerance can be described in a worm in the same way as it is described in humans as being a decrease in the response to a given concentration of ethanol after prior exposure to ethanol.

Wild-type (Bristol strain, N2) *C. elegans* have been shown to exhibit a slight acute tolerance to ethanol over a 50 minute time-span (Davies et al., 2004a) based on recovery from a reduction in speed. The same paper showed that the Hawaiian strain CB4856 exhibited a much greater acute tolerance to ethanol over the same period of time. They demonstrated that this difference was due to the fact that the CB4856 strain carries a lower function 215F allele of the *npr-1* gene compared the higher function 215V allele in N2. These alleles are also the cause of the difference in feeding behaviour between the two strains with N2 being a solitary feeder and CB4856 a social one (de Bono and Bargmann, 1998). However it was shown that these effects probably occur in different neurons (Davies et al., 2004a).

1.11.5 Withdrawal

Withdrawal symptoms are negative effects that occur on cessation of alcohol use. In humans these include tremors, sweats, insomnia and seizures. In worms this could be investigated by looking for a difference in behaviour between control worms and worms that have been exposed to chronic ethanol and then removed from ethanol.

Only one study has looked at a withdrawal effect on *C. elegans*. They showed that after an 18-22 hour exposure to 350mM ethanol, N2 animals show clumping and bordering behaviours when removed from ethanol (Davies et al., 2004a). This is when animals aggregate on the edges of the bacterial lawn, where the bacteria are thickest,

rather than spreading all over the lawn and feeding in a solitary manner. It is also called social feeding. N2 are normally solitary feeders. Social feeding is a phenotype associated with lower function of the *npr-1* gene (de Bono and Bargmann, 1998). They also showed that mutations that suppress the clumping phenotype associated with *npr-1* loss of function also suppress the clumping due to ethanol withdrawal. They suggested that this, and their previous results showing that lower function of *npr-1* leads to increased ethanol tolerance, indicated that long term exposure to ethanol downregulated the NPR-1 pathway, which was presumably activated by acute exposure to ethanol. They then further demonstrated that acute ethanol could suppress the social feeding phenotype in *npr-1* animals, indicating that it was indeed activating this pathway downstream of NPR-1.

1.11.6 Relief from withdrawal

However, chronic exposure to ethanol may have various effects on *C. elegans* which would persist in the absence of ethanol and yet could not be considered to be neuroadaptation to ethanol. A recent paper described chronic exposure to ethanol as resulting in a developmental delay, decreased fecundity, longevity and pharyngeal pumping, when exposure occurred during larval development, and in reduced body length, decreased fecundity and a shorter life expectancy, when exposure occurred during adulthood alone (Davis et al., 2008). It is entirely possible that these effects would cause a difference in behaviour between control worms and worms that have been exposed to chronic ethanol and then removed from ethanol, without any neuroadaptation having occurred.

In addition in mammalian systems ethanol has been shown to cause a dose-dependent increase in the production of reactive oxygen species and a dose dependent increase in

heat shock protein levels (Russo et al., 2001). In *C. elegans* as well exposure to ethanol has been shown to cause upregulation in heat shock protein genes (Kwon et al., 2004). Thus the activation of cellular stress pathways could produce an effect of ethanol conditioning that persisted after removal of ethanol

Other chronic effects of ethanol could include, as a result of the reduction in pumping rate seen in acute intoxication, a food deprivation effect. Any of these chronic effects of ethanol may cause behavioural changes that persist after ethanol removal and could thus be confused with ethanol withdrawal.

In order to conclusively demonstrate a withdrawal effect that is a result of neuroadaptation to ethanol, it will therefore be necessary to demonstrate that the withdrawal behaviour is reduced when ethanol is reapplied. If the withdrawal behaviour is the result of adaptations that counter the effects of ethanol, the renewed presence of ethanol will counter the withdrawal effect. Preferably the withdrawal behaviour would be reduced in response to a low concentration of ethanol, in order to avoid confusion with the effects of ethanol intoxication. This reduction in withdrawal behaviour in response to a low concentration of ethanol is described as relief from withdrawal in the rest of this thesis.

1.11.7 Summary

	No ethanol	**Low dose ethanol**	**High dose ethanol**
Not previously exposed to ethanol	Sober – normal	Slight disinhibition – personality changes, relief from anxiety	Intoxication - ataxia, motor impairment, sedation
After chronic ethanol exposure	Withdrawal – hyperexcitability, anxiety, negative affective state	Relief from withdrawal	Tolerance – reduced susceptibility of acute effects of alcohol

Table 1.5 Summary of inter-related behavioural states induced by alcohol.

1.12 Aims of the project

- To demonstrate intoxication, tolerance, withdrawal and relief from withdrawal in *C. elegans* using the definitions described above.
- To investigate in more detail which behaviours are affected by this neuroadaptation to ethanol in *C. elegans*.
- To explore which major transmitter pathways and neuromodulators are essential for this process, using either a forward genetic screen or reverse genetic disruption of potential pathways (described in Table 1.4).

Chapter 2 - Materials and Methods

2.1 *C. elegans* techniques

C. elegans were cultured according to standard protocols (Brenner, 1974) as described below.

2.1.1 *C. elegans* culture on Nematode Growth Medium (NGM)

C. elegans was cultured on Nematode Growth Medium (NGM) plates which were poured using a plate pouring machine (Jencons Scientific Ltd) to a final volume of approx 10ml per 6cm petri dish. NGM plates had *E. coli* 50µl OP50 added to them as a food source (seeding) and were then left for at least two nights at 20 ±4°C in order for the OP50 to multiply before having *C. elegans* added to them.

C. elegans were maintained at 20°C in an incubator on plates sealed with parafilm to prevent cross-contamination of strains. Unless otherwise stated *C. elegans* used for any experiments were picked as larval stage 4 (L4) animals the night before these experiments and so were young adults (L4 +1 day) at the time of the assay.

2.1.2 Strains and alleles

The standard laboratory N2 Bristol strain was used as a wild type reference. Strains were obtained from the *Caenorhabditis* Genetics Centre (CGC), except for *slo-1 pd24* and *slo-1 pd23* which were originally obtained by Marcus Guest in a screen for resistance to the anthelmintic drug, emodepside (Guest et al., 2007) and then outcrossed (*pd23* outcrossed 3x, *pd24* outcrossed 2x).

Strains used were:

Strain	Gene	Allele	Mutation
N1968	*slo-1*	*js379*	Nonsense Q251>stop In fourth transmembrane domain
XA3747	*slo-1*	*pd23*	In RCK domain
XA3748	*slo-1*	*pd24*	In RCK domain
AX201	*npr-1*	*ky13*	Nonsense Q61>stop
CB4856	*npr-1* among others	wild type Hawaiian strain	V215F in *npr-1* but many other SNPs present
XA3741	*egl-3*	*ok979*	1578bp deletion
CB156	*unc-25*	*e156*	Not known
CB407	*unc-49*	*e407*	Nonsense Q179>stop
CB1112	*cat-2*	*e1112*	Nonsense Q211>stop
MT6308	*eat-4*	*ky5*	300bp deletion
GR1321	*tph-1*	*mg280*	deletion

Table 2.1 *C. elegans* strains used in this thesis.

2.1.3 Removal of contaminants by bleaching

Strains contaminated with bacteria or fungi were cleaned by bleaching as detailed below. This procedure was also used, where indicated, to obtain an age-synchronised population of *C. elegans*.

Gravid adults were washed off plates in 1ml of M9. Worms were left for five minutes to settle before the supernatant was removed. 100µl of bleach mixture (see section 2.12.2 for composition) was added to the pellet. After one minute 1ml of M9 was added to the bleach mixture and the mixture was centrifuged at 13000rpm for 20s. The supernatant was removed and 1ml of M9 was added. The mixture was gently shaken before being centrifuged again at 13000rpm for 20s. The majority of the supernatant was removed leaving approximately 100µl in the eppendorf. The worm pellet was mixed up in this and pipetted around the edge of a clean, seeded (see section 2.1.4) NGM plate. The age-synchronised L1 develop in the seeded OP50.

2.1.4 Maintenance of OP50

E. coli OP50 is a uracil auxotroph whose growth is limited on NGM plates. A limited bacterial lawn is desirable because it allows for easier observation and better mating of the worms. *E. coli* OP50 was grown up from frozen stocks once a year. It was otherwise passaged on LB agar stock plates onto which it was streaked to produce many colonies. For use, an individual colony was picked from the LB plate and used to aseptically inoculate LB broth. This culture was grown up overnight at 37°C. It was then used to seed NGM plates. To seed a plate 50µl of this culture was added to the centre of the agar plate under sterile conditions. If not needed immediately, the culture could be kept at 4°C for up to a month.

2.2 Measurement of ethanol concentration in *C. elegans*

The internal ethanol concentration of young adult animals immersed in 500 mM ethanol for 20 min was estimated according to the published method (Davies et al., 2003). For each assay, approximately 500 young adult worms were washed off a plate in Dent's saline (500 µl) and dispensed into an Eppendorf tube. The worms were left to settle and the supernatant was then removed. The worms were washed twice in Dent's (500 µl) to remove any adhering bacteria. The worms were then re-suspended in 500mM ethanol (500 µl) and maintained at ~20°C for 20 minutes. The worms were centrifuged at 4°C (1600g; 30s). The supernatant was carefully removed. For one set of experiments the worm pellet was not washed, in the second set of experiments they were re-suspended in 50 µl ice-cold distilled water and for the third set of experiments they were re-suspended in 500 µl ice-cold distilled water. All the samples were then centrifuged, the supernatant removed and the pellets re-suspended in 40 µl ice-cold distilled water. The volume was estimated by visual comparison with calibrated tubes.

The worms were then lysed by four freeze-thaw cycles in liquid nitrogen and sonication. The samples were then centrifuged at 4°C and the supernatant removed for analysis. The supernatant was analysed in triplicate, both undiluted and with a single five-fold dilution. The ethanol concentration in 10 µl aliquots of these samples was determined using a Randox Blood Alcohol Kit (see section 2.3.5).

Alternatively to check the effect of increasing durations of the wash step on the assay described above the experiment was performed as above with the following exceptions. The worms were added to 1M ethanol rather than 500mM. Five sets of experiments were performed. In one set the worm pellet was not washed. In the other four the pellets were washed with 50µl ice-cold distilled water. This was either removed as quickly as possible (1 minute wash) or left in contact with the pellet for increasing amounts of time (2, 3 and 4 minute washes) before the supernatant was removed.

2.3 Preparation of ethanol plates and solutions

2.3.1 Preparation of ethanol solutions

All ethanol solutions were prepared on the same day that they were used in order to minimise loss of ethanol by evaporation. Ethanol solutions were made up in either distilled water, M9 or Dent's saline.

2.3.2 Preparation of ethanol plates for acute behavioural assays

Agar plates containing ethanol but no food were required to measure the rate of body bends on agar in the presence of ethanol (see section 2.5.2). This assay was performed as part of two different experiments and the ethanol plates were prepared differently.

All NGM agar plates were poured using a plate pouring machine (Jencons Scientific Ltd) to ensure a uniform volume of approx 10ml and left to set for at least one night.

For the first experiment the rate of body bends on agar in the presence of ethanol was measured in order to compare this with the rate of thrashes in liquid in the presence of ethanol. Thus the agar plates needed to be at the same exact concentrations of ethanol as was the liquid. Thus agar plates from one batch of plates had a range of standard ethanol volumes added to them, were sealed with parafilm, left overnight to equilibrate and tested for ethanol concentration in the morning. A calibration curve was made and from this, agar plates were made in the concentration range 10-500mM ethanol. These plates were from the same batch and were sealed with parafilm and left overnight to equilibrate.

The second experiment measured the rate of body bends on acute ethanol after conditioning. In this ethanol was added to the plates to make a final concentration of approximately 250mM and the plates were sealed with parafilm and left overnight to equilibrate before the assay. The exact ethanol concentration of the plates was then measured after the assay.

2.3.3 Preparation of conditioning plates for chronic ethanol treatment

Conditioning plates for chronic ethanol treatment were made in such as way as to control the environmental conditions as precisely as possible to minimise variation between assays. All NGM agar plates were poured using a plate pouring machine (Jencons Scientific Ltd) to ensure a uniform volume of approx 10ml and left to set for

at least one night. Fresh OP50 was made up and left overnight at 37°C. The next day the optical density of this OP50 was measured and it was diluted to 0.8A OD600 and 50µl was added to 6cm agar plates (conditioning plates). These were left for 2 days in order for the OP50 to grow and then ethanol was added to some of them and the plates were sealed with parafilm. For most experiments 0.21ml ethanol was added to half the conditioning plates (to make approximate final concentration of 250mM) and no ethanol was added to the other half as a control. For the food race using lower conditioning concentrations the volumes used were 0ml, 0.026ml, 0.105ml and 0.21ml (to make approximate final concentrations 0mM, 50mM, 150mM, and 250mM). *C. elegans* were added to the plates the day after to allow time for the ethanol to equilibrate. For 48 hour conditioning experiments the ethanol concentration of the plates was measured before and after the assay and an average concentration taken. For 6 hour conditioning experiments ethanol concentrations were measured after the assay.

2.3.4 Preparation of test ethanol plates for food race and video capture assays

9cm agar plates were poured using 25ml NGM agar per plate (test plates). The next day the optical density of the OP50 was measured and diluted to 0.8A OD600 and 50µl of this was added 2cm from the edge of the test plates. The next day ethanol was added to the test plates and the plates were sealed with parafilm. Volumes of ethanol added to the test plates for the initial food races were 0ml, 0.07ml, 0.28ml and 0.56ml (control, low, medium and high ethanol – approximate final concentrations of 0mM, 50mM, 150mM and 250mM). For the videos only control, low and high ethanol plates were made. For some later food races only control and low ethanol plates were

made. Low ethanol plates were used to demonstrate relief from withdrawal. Ethanol concentrations were measured after the experiment.

2.3.5 Ethanol estimates

Two assay kits were used to estimate the concentration of ethanol in samples. Initial tests were done using a Randox Blood Alcohol Kit according to the manufacturer's instructions except that each of the components was used at a tenth of the recommended volume. However the production of the Randox Blood Alcohol Kit was discontinued. So for later experiments the NAD-ADH Reagent Multiple Test Vial from Sigma-Aldrich was used where indicated according to the manufacturers instructions except that 0.6ml of reagent was added to 0.02ml of sample instead of 3ml reagents added to 0.1ml of sample.

Both of these assay kits contain alcohol dehydrogenase (ADH), nicotinamide adenine dinucleotide (NAD) and a buffer. They work on the principle that in the presence of ADH;

$$\text{Ethanol} + \text{NAD} \leftrightarrow \text{acetaldehyde} + \text{NADH}$$

NADH absorbs light at 340nm with an extinction coefficient of 6.2 mM^{-1} cm^{-1}, and so the relative concentration of this can be measured using a spectrometer. The absorbance is measured after a fixed period of time. The absolute concentration of ethanol in the samples can thus be obtained using a calibration curve.

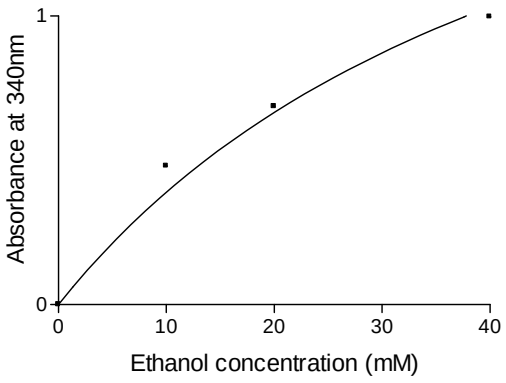


Figure 2.1 Example calibration curve for an ethanol concentration test. Samples are diluted into the sensitive range.

To analyse the data GraphPad Prism was used to draw a calibration curve using non-linear regression. From this the ethanol concentration of the samples was calculated.

2.3.6 Measuring ethanol concentration in agar

The concentration of ethanol in the agar plates was then measured thus. A $1cm^2$ square of agar (to ensure approximately similar amounts) was cut out from the centre of each plate and weighed (to enable calculation of its dilution). A sample of three plates was measured for each concentration used. This square of agar was then added to 1ml of distilled water in an eppendorf and sonicated for an hour in order to allow the ethanol to equilibrate between the water and the agar. Three 10 μl aliquots of liquid were taken from each eppendorf and the ethanol concentration in each of these samples was determined (see section 2.3.5). The ethanol concentration of the original agar plates is then determined by calculating the original dilution of the agar in the 1ml distilled water.

tube number	Absorbance	contents	average controls	samples minus controls	Ethanol conc (mM)	Cube volumes	Real ethanol conc (mM)		Average
26-Sep	arbitrary units				(from calibration curve)				
example calculation			AVERAGE(B4:B6)	B7-D4			F6*((1+G6)/G6)		AVERAGE(H7:H18)
1	0.147	conditioning control	0.149						
	0.141								
	0.159								
2	1.191	conditioning ethanol		1.042	64.26	0.39	229.03		207.77
	1.191			1.042	64.26	0.39	229.03		
	1.199			1.050	65.08	0.39	231.94		
3	1.269	conditioning ethanol		1.120	72.57	0.59	196.20		
	1.309			1.160	77.17	0.59	208.64		
	1.296			1.147	75.65	0.59	204.53		
4	1.135	conditioning ethanol		0.986	58.77	0.50	177.02		
	1.181			1.032	63.25	0.50	190.52		
	1.173			1.024	62.45	0.50	188.12		
5	1.123	conditioning ethanol		0.974	57.64	0.39	205.06		
	1.165			1.016	61.66	0.39	219.37		
	1.149			1.000	60.11	0.39	213.84		

Figure 2.2 Example calculation of ethanol concentration.

2.4 Conditioning *C. elegans* with ethanol

Worms were conditioned with ethanol for various experiments. Conditioning plates were made as described in section 2.3.3. Worms were conditioned (or otherwise kept under the same conditions in the absence of ethanol) for either 48 or 6 hours and then washed to remove residual ethanol before being used in an assay. Depending on the assay worms were either washed as a population or individually.

Assay	Food race	Food race (6 hour)	Videos	Pumping/ Body bends/ Development	Egg laying
Length of conditioning time (hours)	48	6	6	48	48
Type of wash	Population	Population	Individual	Individual	Individual

Table 2.2 Conditioning procedures used in this thesis

For 48 hour conditioning worms were picked as L4 onto conditioning plates with no more than 50 L4 on each plate so that the food was in excess. For 6 hour conditioning worms were picked as L4 the day before the assay onto fresh plates, which contain food but no ethanol. On the morning of the assay the worms were washed off these plates in M9 with 0.1% Bovine serum albumin (BSA), the supernatant removed, and the worms added to the conditioning plates (with food) in a small volume of M9. In both cases the conditioning plates were sealed with parafilm after the worms had been added to them and placed in an incubator at 20°C for the duration of the conditioning period.

Where worms were washed as a population, after conditioning and prior to the assay the worms were washed off the conditioning plates in M9 with 0.1% BSA. After settling, the supernatant was removed, and worms resuspended in 1ml M9 and left for two minutes. This was repeated to remove residual ethanol (see section 2.11.1). Finally worms were pipetted onto the assay plates in a small volume (30µl) of M9 with 0.1% BSA.

Where worms were assayed individually, worms were picked off the conditioning plates one by one into a large volume (3ml) of M9 with 0.1% BSA. They were left for at least two minutes in order to remove residual ethanol. They were then pipetted out of the M9 solution onto an unseeded plate and left for a minute so as to remove residual liquid, before being picked onto the test plate.

2.5 Behavioural assays

2.5.1 Thrashing assays

In liquid, wild type animals exhibit a rhythmic flexing motion centred on the midpoint of the body called "thrashing". A single thrash is defined as a complete movement through the midpoint and back. A thrashing assay measures the number of thrashes a worm makes in a given period of time. All assays were performed on young adult animals (L4 + 1 day), in a temperature-controlled room at 20^{o}C.

Single worms were placed in an embryo dish, containing 1ml Dent's saline. The basal thrashing rate of each worm in the absence of ethanol was recorded. 3ml of a solution of ethanol in Dents saline was then added to the 1ml Dents saline to bring the ethanol concentration to the final desired concentration. The dishes were topped up with ethanol solution of the required concentration until they were full to the brim. The dish was then sealed with a glass lid to prevent evaporation of ethanol. Alternatively, as a control, the dishes were filled completely with Dent's saline and sealed. The number of thrashes in a 30 second period was then counted either every minute or every five minutes, as indicated

The rate of recovery from ethanol intoxication was investigated using the thrashing assay. The number of thrashes per minute of each worm in 1ml Dent's saline was recorded to give a basal thrashing rate for each worm. The dishes were then filled and sealed as before, with ethanol solution or Dent's saline alone (control). After ten minutes, the number of thrashes per minute was recorded to give a rate of thrashing in ethanol (or control) for each worm. The worm was then removed, with minimal

ethanol, using a Gilson pipette and placed in a watch glass containing a large excess of Dent's saline. The number of thrashes in a 30 second period was recorded immediately, and every minute for ten minutes, for the ethanol treated animals and for the Dent's saline controls.

2.5.2 Body bends assays

On an agar plate a worm moves with a rhythmic sinusoidal motion. One body bend is defined as the area just behind the pharynx bending in the opposite direction and then returning to its original direction. In a body bends assay the number of body bends a worm makes in a given period of time is counted. All assays were performed at room temperature (approx 20-22°C). All assays were performed on agar plates in the absence of food. All assays were performed on worms, which had spent a minute on a fresh non food 'cleaning' plate to remove bacteria immediately prior to the assay.

Where the rate of body bends in the presence of ethanol was measured, the method for making the ethanol plates is described in section 2.3.2. Where the rate of body bends after conditioning was measured, the method for conditioning and washing the worms is described in section 2.4.

2.5.3 Visual determination of pumping rate

A worm's feeding behaviour consists of rhythmical contractions of the pharynx, drawing bacteria up the isthmus and into the terminal bulb where they are crushed by the grinder. This is called pumping. The movement of the grinder is visible under the microscope and thus the number of pumps/minute can be measured visually. All assays were performed at room temperature (approx 20-22°C). All assays were

performed on a fresh food plate. The worms were left on the food plate for five minutes and then the number of pumps occurring in a minute was recorded.

The pumping rate after conditioning was recorded. Worms were conditioned and washed as described in section 2.4.

2.5.4 Egg laying assay

Worms were conditioned and washed as described in section 2.4. 10 worms were conditioned per ethanol plate and 10 per control plate. These worms were then placed on fresh food plates. The number of eggs on the conditioning plates was counted to record to egg laying rate during intoxication. After 24 hours the number of eggs on the fresh food plates was also counted to record the egg laying rate during withdrawal.

2.5.5 Food race assays

For the food race assay 100 worms were washed off plates in M9 buffer with 0.1% BSA. The conditioned worms were conditioned and washed as described in section 2.4. For the unconditioned assay worms were picked as L4 the night before the assay onto fresh food plates (100 worms per plate) and they were then washed in the same manner to remove bacteria. They were then added to the test plates (see section 2.3.4) 2cm in from the edge on the opposite side to the food in 30µl M9.

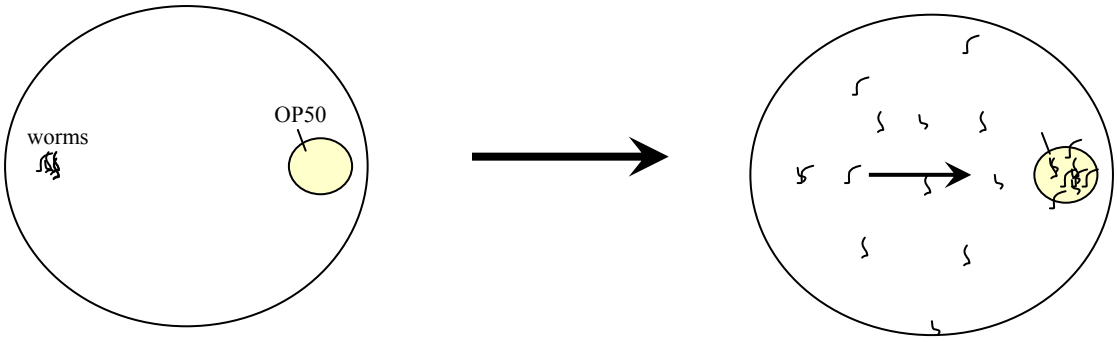


Figure 2.3 Diagram illustrating the food race assay

After M9 had evaporated the number of worms that had reached the food were counted every ten minutes and subsequently removed. After two hours the total number of worms left on the plate was counted and used to calculate the percentage of animals that had reached the food at each time point. The cumulative percentage of worms reaching the food per unit time was plotted.

For recovery from conditioning experiments some of the animals were run on a food race as normal and the others washed as described and left on fresh food plates for either 6 or 24 hours before being washed to remove bacteria and placed in another food race.

2.5.6 Measurement of the area of a worm

In order to optimise the conditioning assays it was investigated if conditioning affected worm development. Worms were conditioned and washed as described in section 2.4. Worms were then placed onto fresh food plates and photographed using the same magnification for each picture. Using SimplePCI software program the shape of the worm was defined using intensity (as the worms were darker on the picture than the surrounding agar and OP50) and the area (in pixels) that the worm took up on the photograph measured. This method was used to compare the average areas of five conditioned worms to five non-conditioned worms in order to ascertain if conditioning had affected the worm's development.

2.5.7 Aldicarb assays

Aldicarb plates were made to a 0.5mM final concentration of aldicarb. Two different experiments were performed using aldicarb assays. One measured the effect of acute

ethanol in the aldicarb plate on the rate of paralysis by aldicarb. For this aldicarb was dissolved in ethanol. The other measured the effect of ethanol withdrawal on the rate of paralysis by aldicarb. For this aldicarb was dissolved in DMSO. In both cases the aldicarb was added to the liquid agar before pouring the aldicarb plates. The plates were poured three days prior to the assay. The plates were seeded with *E. coli* OP50 at an optical density of 0.8A OD600, two days prior to the assay. The day before the assay ethanol was added to some of the aldicarb plates used in the acute ethanol assay to make estimated final concentrations of 100, 200 and 300mM ethanol in the aldicarb plates. The exact final ethanol concentration was measured after the assay. Plates used in the ethanol withdrawal assay contained no ethanol at all. *C. elegans* were picked as L4 the day before the assay and so were young adults on the day of the assay.

All aldicarb assays were performed blind. 20 worms per plate were added to the aldicarb plates. Paralysis was described as being when a worm moved neither backwards nor forwards in response to nose touch. Every half hour the number of paralysed worms was recorded and the paralysed worms were picked off the aldicarb plate. The assay continued until all worms were paralysed.

Vehicle controls were performed in which all conditions were identical except for the absence of aldicarb. In these the number of paralysed worms was measured every half hour for three hours.

2.6 Isolating DNA for sequencing

2.6.1 DNA extraction

DNA was extracted from a population of well fed worms. These were washed off an agar plate in 1ml M9 and washed again in M9. The supernatant was replaced with 100µl of worm-lysis buffer containing proteinase K (100ng/ml). The mixture was then frozen at -80°C for 15 minutes to lyse the worms by freeze-thaw, placed in a heat block at 60°C for an hour to allow lysis and degradation of protein. Finally it was heated to 95°C for 15 minutes to denature the proteinase K. 200µl ddH_2O was then added and the mixture was stored at -20°C.

2.6.2 Polymerase Chain Reaction (PCR)

PCR reaction mixture 250µl

dNTPs 3.5 µl
DNA 25 µl
DNA Taq polymerase (expand long template PCR) 1.25 µl
PCR buffer (expand long template PCR) 25µl
Forward primer (10 µM) 7.5 µl
Reverse primer (10 µM) 7.5 µl
ddH_2O 180.25 µl

Cycling conditions

	Temperature	Times	Cycle number
Initial denaturation	94 ^oC	2 minutes	1 x
Denaturation Annealing Elongation	94 ^oC ~ 55 ^oC 68 ^oC	15-30 seconds 30-60 seconds ≤ 20 minutes	15-30 x
Final elongation	68 ^oC	7 minutes	1 x

Table 2.3 Cycling conditions for PCR

The fragments of DNA amplified by PCR were run on an agarose gel with a DNA ladder to determine their size. Loading buffer (5X) was added to the PCR reaction mixture. The agarose gel consisted of 0.8% agarose made up in TBE buffer and 1µl of ethidium bromide per 100 ml. The PCR product was purified using a PCR purification column (Qiagen) according to the manufacturer's instructions. A second round of PCR was performed using a second set of primers complementary for sequences within the amplified fragment.

2.6.3 Sequencing

Sequencing was used to confirm the presence of the expected mutation in the strain of interest. For economic reasons, both in time and money, sequencing was done out of house by MWG Biotech. Dry amplified DNA 20ng/100bp samples and primers at 10mM were sent to the company. Primers are located between 300 and 400 base pairs apart on the cDNA sequence. The concentration of DNA in samples was measured using the Nanodrop spectrometer according to the manufacturer's instructions. The sample was then dried by lyophiliser.

2.6.4 Primers

Name	Sequence	Use
Gap 3 OF	TCAAATTGAAGCTGGAAACG	Outer forward primer for *slo-1 js379* cDNA amplification
Gap 8 OR	TATGGGTGTCAAATTTACGG	Outer reverse primer for *slo-1 js379* cDNA amplification
Gap 3 IF	AGAACCGAGTGAGTTTGATG	Inner nested forward primer for *slo-1 js379* cDNA amplification
Gap 8 IR	AAGTCGCATAACTCAGTCAG	Inner nested reverse primer for *slo-1 js379* cDNA amplification
Sequencing primer	ATCTTAAAATCGCACGGATA	Sequencing primer – to confirm presence of mutation in *slo-1 js379*

Table 2.4 Primers used in this thesis

2.7 Mutagenesis of *C. elegans*

6 plates of N2 worms were grown up so as to contain a large population of mixed stage *C. elegans*. Each plate was washed with 1ml M9 buffer into a 20ml universal tube. Worms were left to settle for 15 minutes and then most of the supernatant was removed. M9 was added to make the contents up to 20ml, worms were allowed to settle for 15mins and then most of the supernatant was removed, leaving 2ml.

All procedures after this point were carried out in a dedicated tray in a dedicated fume cupboard, using a dedicated Gilson to avoid ethylmethanesulphonate (EMS) contamination. Double gloves were worn and were rinsed in 1M NaOH before disposal. Tips were placed in 4M NaOH before disposal. Everything used was bathed in 1M NaOH for 24 hours after the experiment in order to hydrolyse the EMS.

Another 2ml of M9 was measured into a separate tube. 20µl of liquid ethylmethanesulphonate (EMS) (100%) was added to this. This mixture was then added to the tube containing the worms. This tube was then sealed and left on its side for 4 hours, during which time it was rocked gently every half hour.

After this time the 4ml was made up to 20ml with M9. This was left to settle for 15mins. The supernatant was removed leaving 2ml. This was repeated four times. After this the supernatant was removed. The worms were mixed by gentle pipette mixing and 4 x 0.5ml was transferred to individual fresh plates seeded with OP50 for food. After 1 hour 150 individual L4 worms that had reached the food were picked to individual plates. These were the F0 generation.

These F0 worms were allowed to grow to adults and lay eggs for two nights before the adults were removed. The eggs, which formed the F1 generation, were grown up and allowed to self fertilise until they were gravid adults and then bleached to produce an age-synchronised F2 generation which could be grown up and screened for worms showing a reduction in withdrawal.

2.8 Electropharyngeogram recordings

The activity of the pharyngeal muscle was measured using electropharyngeogram recordings (EPG) as described previously (Papaioannou et al., 2005). This detects the electrical transients associated with the rapid contractions and relaxations of the pharyngeal muscle. All experiments were carried out at room temperature (approximately 20±4°C). Recordings of the activity of the pharyngeal muscle were made via a borosilicate glass suction pipette filled with Dents saline applied to the mouth of the animal. This suction pipette was pulled from a borosilicate glass capillary of dimensions 1mm outside diameter and 0.58mm inside diameter using a Narishige Model PB-7 puller. The suction pipette was connected to an Axoclamp 2B-recording amplifier. Data were acquired using Axoscope (Axon Instruments). Recordings were made from intact well fed, young (L4 + 1 day) adult hermaphrodites which were placed in the recording chamber and the suction pipette applied to the mouth. The recording chamber was cut from Sylgard silicone elastomer and placed on a glass slide sealed with silica gel supported on a plastic base. This was viewed using an Olympus phase contrast inverted microscope.

Experiments were performed on both intact worms and dissected worms. In the experiments with intact worms, 1mM 5-HT was included in the Dent's saline to drive

a basal pumping rate against which changes in pump rate could be observed. Ethanol was applied to the preparation by manually exchanging the Dent's saline surrounding the preparation with saline containing ethanol by pipette.

Recordings were also made from dissected animals in which a cut was made just posterior to the pharynx to expose the pharyngeal muscle. These experiments were performed under two different conditions. Unless otherwise stated the exposed pharynxes were perfused with Dent's saline at a net rate of 4ml min^{-1} in order to stimulate basal pumping. Ethanol was applied to the preparation via a semi-sealed perfusion system (net rate 4 ml min^{-1}).

However, in some experiments (as indicated in figure legend), the exposed pharynx preparation was not perfused. This was in order to mimic the situation used in the intact preparation. In this case ethanol was added manually as described above for the intact preparation. In these experiments 50nM 5-HT was included in the saline to drive a basal pumping rate against which the effects of ethanol could be measured.

2.9 Analysis

Behavioural assays were analysed using unpaired Student's t-tests or ANOVA where indicated.

2.10 Video analysis

2.10.1 Video capture for the automated analysis

Worms were conditioned and washed as described in section 2.4. They were then picked onto test food race plates made as described in section 2.3.4. After five

minutes a 30 second video was recorded of the behaviour of the worm without moving the agar plate. Video recordings were taken using a dissecting microscope attached to a Hamamatsu C4742-95 camera and using SimplePCI video recording software. All videos were taken at the same magnification and were converted into .avi files at 2x normal speed.

2.10.2 Automated video analysis

The automated video analysis was carried out using a software package written in Matlab by Christopher James, (ISVR, University of Southampton). Each video is a grey-scale .avi video file containing up to 150 frames showing a single worm moving on agar at a consistent magnification. Each frame is a rectangle of 1024x1280 pixels, each pixel of which has been assigned a value for intensity, which describes its colour along a grey scale between black (0) and white (255).

Extraction of the background image

The initial assumption was that the worm was the only thing moving in the video. Thus to extract the background the mean image was taken of the 150 frames. Every pixel has a value for its intensity in each of the 150 frames. The average of these values was assigned to that pixel to create an average image. The worm will be much darker (lower intensity) than its surroundings. But, as it moves around, it will be averaged out of the background image.

The operator is then shown the background image and asked if this is correct. If the worm has remained stationary it will still be visible. If this is the case the operator can draw a rectangle around the area where the worm is still visible.

The program will then draw a histogram of the intensity values within this rectangle. There will be two peaks on the histogram, one corresponding to the worm and one to the background. The program will calculate the median intensity value and replace all the intensity values below the median with the median value. This will remove the worm from the background image. The operator is then shown the new background image and asked if this is correct. This process can repeat until the operator is satisfied.

Creating a binary image of the worm

From this point the program works on a frame by frame basis. The background image is subtracted from each frame. Pixels that contain the background should thus have an intensity value close to zero. All pixels with intensity values that are within a certain range of zero are assigned the value zero (black). All pixels with intensity values beyond this threshold are assigned the value one (white). A binary image of the worm has thus been produced.

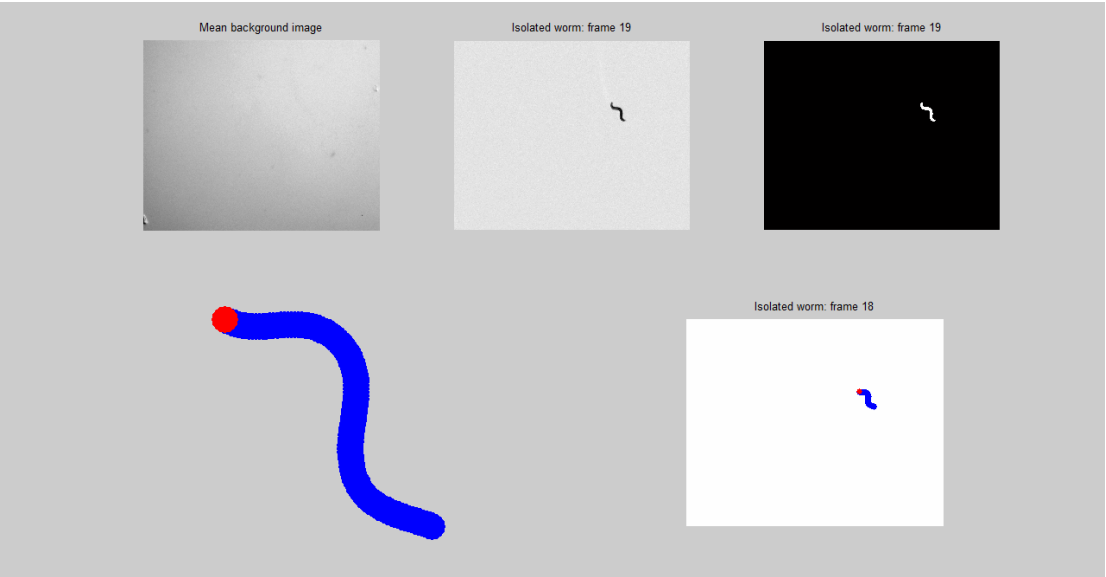


Figure 2.4 Process of analysis of a video. Clockwise from top left; the mean background image, one frame with the background deleted, the binary image of the same frame and lastly, also from the same frame, the best fit curve between the ten nodes with node 1 (the head) marked in red.

Building a parameterized worm model

Again this was performed on a frame by frame basis. The parameterised worm was described using a Gaussian mixture model (GMM). The frame of the video being analysed can be described as a 3D graph with the co-ordinates of each pixel in the 2D frame along the x and y axes and the intensity of that pixel on the z axis. This produces a distribution which cannot be easily described statistically. This is modelled using ten Gaussian distributions, which are well characterised statistically. Each Gaussian can be described by its mean (x and y coordinates), amplitude (intensity) and variance. In order to model the worm using these ten Gaussians, an expectation maximisation (EM) paradigm is used. This measures the error between the Gaussian mixture model and the actual intensity distribution of the image, alters the parameters of the Gaussians and measures the error again. This repeats until the error converges. This paradigm minimises the error between the model and the real image.

To minimise the number of iterations required, constraints are placed on the amplitude and variance of the distributions and the initial mean coordinates are taken from the final mean coordinates of the previous frame. For the first frame in the video the operator is shown a binary image of the worm from the first frame and asked to mark ten points along its length with the mouse, starting with the head to give the initial coordinates. The ten Gaussians are numbered 1-10 in accordance with the order in which the operator marked them in the first frame, with 1 being the head of the worm and 10 being the tail.

A parameterised worm is drawn by taking the ten mean xy coordinates of the Gaussians (node centres) and joining them with a best fit curve. The head is marked in

red. The xy coordinates of the ten node centres in every frame of the video are then saved.

Calculation of loopyness

A linear regression line is drawn between the ten node centres. This is the straight line which minimises the sum of the squares of the perpendicular distances of each node centre to the line. This is not affected by the ordering of the node centres.

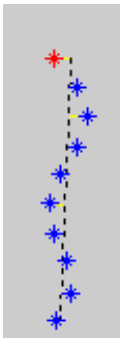


Figure 2.5 Regression line through ten node centres in one frame of a video

The perpendicular distance of each node centre to the regression line is then measured and the ten values averaged. This gives a value for the loopyness of the worm for each frame. The value for every frame in the video can be averaged to give an overall value for the loopyness of the worm.

Calculation of centre of mass of the worm

Each of the ten node centres has an x and a y coordinate. The average of all the x coordinates is the x coordinate of the centre of mass and likewise for the y coordinates. This gives a xy coordinate for the centre of mass of the worm in any given frame. By joining the centre of mass position for every frame in the video a track of the movement of the worm can be drawn.

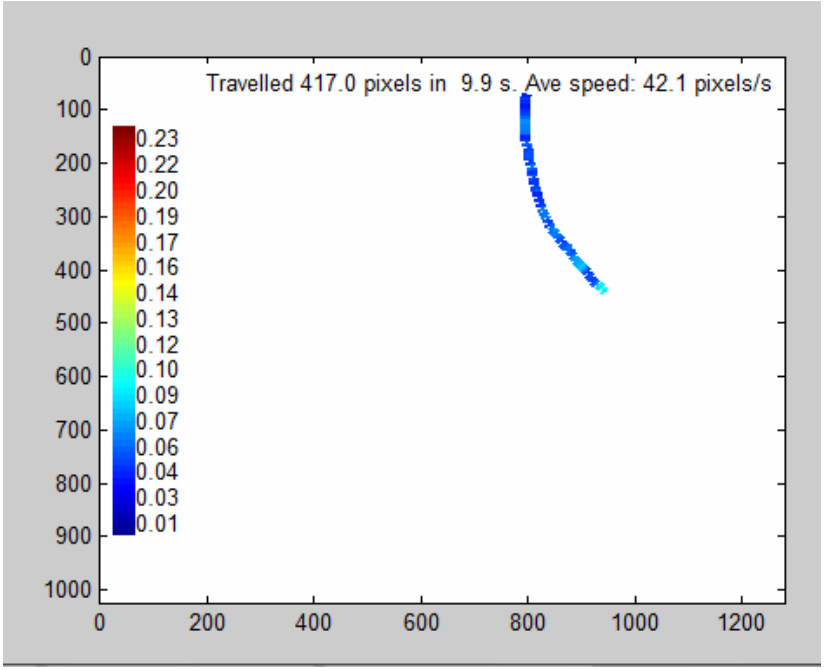


Figure 2.6 Track showing the position of the centre of mass of the worm in every frame of a single video. Colour scale shows loopyness measure.

Calculation of speed

The centres of mass can be used to calculate the distance travelled by the worm between each frame ($x^2 + y^2 = d^2$) and thus the total distance travelled during the video. This distance (in pixels) divided by the duration of the video (in seconds) is the average speed of the worm.

Calculation of efficiency

An alternative centre (centre of worm) is calculated by measuring half the distance of the length of the best fit curve joining the ten node centres, along the best fit curve joining the ten node centres. If the centre of the worm in every frame is joined up to make a track, this can also be used to calculate distance travelled. This produces a larger value as this track follows the sinusoidal movement of the worm.

The efficiency of the sinusoidal worm movement has been calculated as;

$$\text{Efficiency} = \frac{\text{distance covered by centre of mass}}{\text{distance covered by centre of worm}}$$

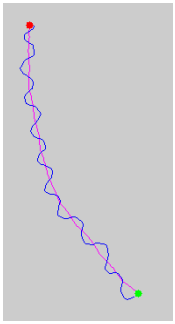


Figure 2.7 Track showing the positions of the centre of the worm (blue) and the centre of mass (pink) in every frame of the video.

Cluster analysis

For every frame of every video there are now ten xy coordinates, one for each node. Their positions relative to each other could be plotted on a ten dimensional graph, so that each frame was a point on the graph. To visualise this data more clearly it needs to be simplified.

The Neuroscale algorithm takes multidimensional data and renders it in a lower dimensional visualisation space. It does this by calculating the Euclidean distance between each two points in the ten-dimensional space and creating the equivalent distance between these points in two-dimensional space. It thus plots a point on a 2D space for every frame of the video in such a way that the ordering and separation of the points is as similar as possible to its ordering and separation in 10D space. It learns this mapping when given a large selection of videos and can then plot back the points representing the frames in one video, or groups of videos onto the positions of all the videos it has seen.

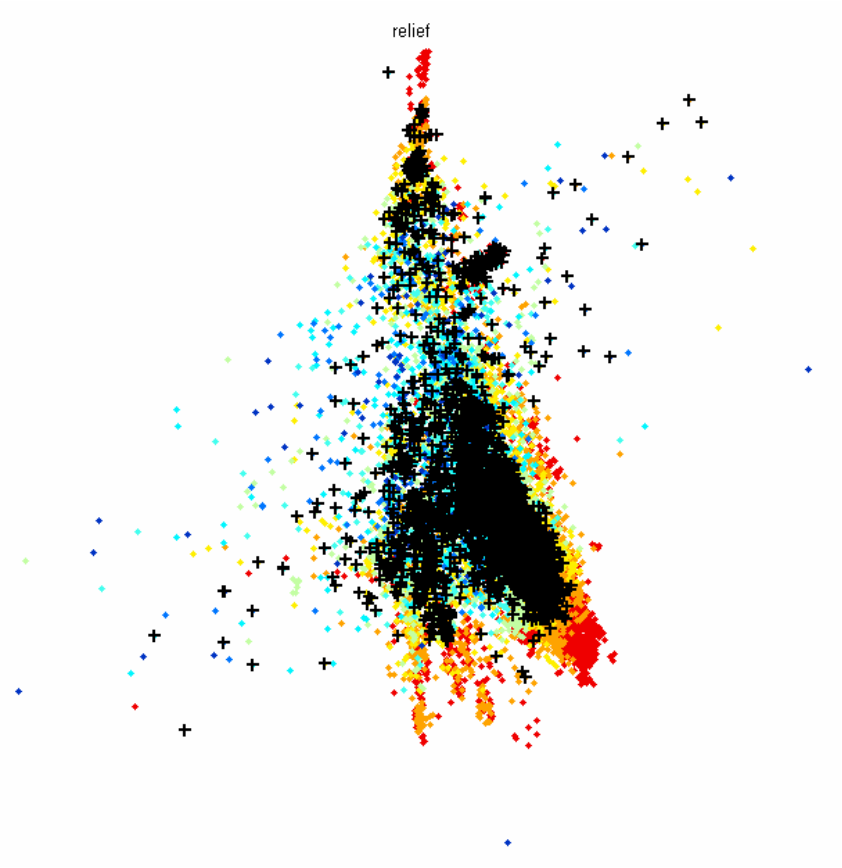


Figure 2.8 Example cluster analysis: the positions of each frame in twenty videos showing relief from withdrawal are plotted in black on a background of 120 videos.

In order to reduce the amount of computational power required to run the Neuroscale algorithm on large data sets k means clustering was used. K means clustering is a standard method of dealing with large data sets. Instead of using the entire data set, groups of similar data points are designated clusters and a cluster centre is determined. These cluster centres are then used for the analysis rather than the data points themselves. This reduces the RAM required to run the cluster analysis to manageable levels.

The cluster analysis itself produces a spread of data points such that points close together represent similar worm shapes and points far apart from each other represent very different worm shapes. By plotting worms under different conditions onto the

cluster analysis one can see if these conditions affect the distribution of body shapes that a worm can display.

2.10.3 Video capture to measure reversals and omega turns

Worms were conditioned and washed as described in section 2.4. They were then picked onto test food race plates made as described in section 2.3.4. After five minutes a five minute video was recorded of the behaviour of the worm. The video was recorded using the equipment described in section 2.10.1. If the worm reached the edge of the field of view the agar plate was moved to bring it back to the centre of the field of view and if the worm had still not reached the food 40 minutes after being added to the plate another five minute video recording was made of it. The videos were converted to .avi files at 2.5x normal speed.

2.10.4 Analysis of reversals videos

For these the videos were analysed by eye, with the time every reversal or omega turn took place, the length of every reversal and the behaviour following every reversal e.g. omega turn, or change of direction, being recorded. See section 1.11.1 for more detail.

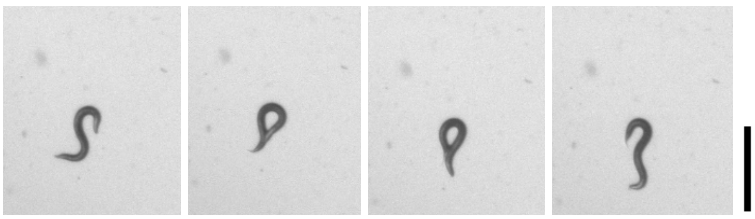


Figure 2.9 Example omega turn. Scale bar represents 1mm.

2.11 Optimisation of assay procedures

2.11.1 Determining the time required to remove residual ethanol from the worm

The time taken to recover from ethanol intoxication was measured using the thrashing assay as described in section 2.5.1. This showed that a worm fully recovered from the effects of ethanol within two minutes (Figure 3.4). Therefore all worms were washed for at least two minutes after ethanol conditioning to fully remove any residual ethanol.

2.11.2 Optimizing the time required for ethanol to equilibrate in an agar plate

In order to optimise the procedure for making ethanol plates it was necessary to investigate how long it took ethanol to equilibrate across the agar plate and whether any ethanol would be lost to evaporation over time. NGM agar plates were poured using a plate pouring machine (Jencons Scientific Ltd) to ensure a uniform volume of approx 10ml and left to set overnight. The following day absolute (99.99%) ethanol was added to the plates (either no ethanol as a control or to three final concentrations of ethanol). The plates were sealed with parafilm and left to equilibrate for 2 hours, 24 hours or 72 hours. The concentration of ethanol in the centre of the plates was then tested (Figure 2.10).

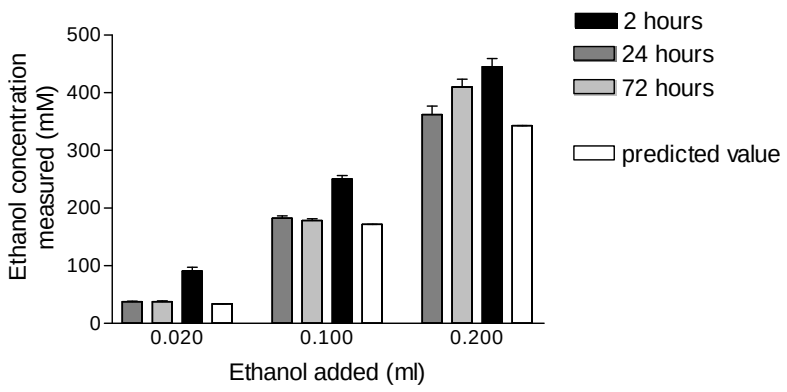


Figure 2.10 Concentrations of ethanol in NGM agar plates measured at 3 time points after adding given volumes of ethanol.

The concentration in the centre of the plate after 2 hours was still higher than expected indicating that the ethanol had yet to fully equilibrate. The concentration in the centre of the plate was stable between 24 and 72 hours indicating that the concentration of ethanol in the plates had equilibrated and was not significantly altered by evaporation. Thus in all experiments involving ethanol plates, ethanol was added to the plates 24 hours before use and the plates were then sealed with parafilm.

2.11.3 Assessing if ethanol in an agar plate affects the worm to the same extent as the same concentration of ethanol in liquid

In order to optimise the behavioural assays on agar plates it was necessary to check that exposure of a worm to ethanol containing agar had quantitatively the same effect as exposing it to an ethanol containing solution. Agar plates containing defined concentrations of ethanol in the range 10-500mM were made as described in section 2.3.2. The percentage decrease in the rate of body bends at defined concentrations of ethanol in this range, relative to the basal rate of body bends was then measured. This was found to be comparable with the percentage decrease in the rate of thrashing in ethanol solution at the same concentrations (see Figure 3.6). There is no significant difference between them measured by two-way ANOVA ($F_{1,298}$=3.214, P=0.074).

This was taken as a further indication that the ethanol plates had been made up to the correct concentration and that placing a worm on an ethanol containing agar plate affected it in the same way as placing it in an ethanol containing solution.

2.11.4 Measurement of the effect of *E. coli* OP50 and *C. elegans* on the concentration of ethanol on agar plates

In order to optimise the procedure for conditioning worms with ethanol it was necessary to investigate if the presence of *E. coli* OP50 (food) or *C. elegans* themselves on an agar plate would affect its ethanol concentration. NGM agar plates made as described above were seeded with 50µl of OP50 at an optical density of 0.8A (OD600). They were then left for 2 days to allow the OP50 to grow. After 48 hours ethanol was added to the plates (either no ethanol or one of three other concentrations of ethanol). The ethanol containing plates were then left overnight to equilibrate. Next, 3 sample plates were tested for ethanol concentration whilst 10 L4 worms per plate were added to the half of the rest of the plates (day 1). After a further 48 hours (day 3) the L4 were one by one taken off the plates for an assay. 48 hours later (day 5) the plates that had contained the worms and OP50 and ethanol were tested for ethanol concentration, as were some more of the plates that had only had ethanol and OP50 on them (Figure 2.11).

Day	-2	-1	0	1	2	3	4	5
Time line	OP50 (food) added to plates		Ethanol added to plates	Sample of plates tested for ethanol concentration. Worms added to half the others		Worms removed for assay		All plates tested for ethanol concentration.

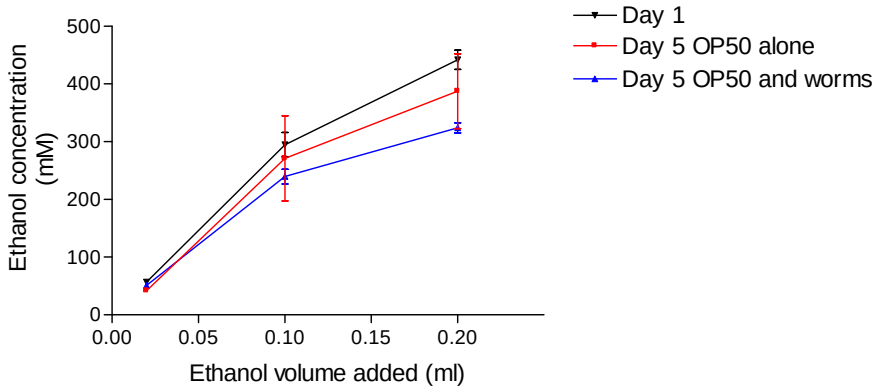


Figure 2.11 Effect of the presence of *E. coli* OP50 and *C. elegans* on ethanol concentration of agar plates. The worms were added to the plates on day 1, 24 hours after the ethanol had been added to plates which already contained a defined amount of OP50.

There is a significant effect of the presence of both OP50 and worms together measured by two-way ANOVA ($F_{1,30}$=13.36, P=0.001) but not of OP50 alone ($F_{1,36}$=0.7331, P=0.3975). Therefore for subsequent experiments using long term conditioning plates the ethanol concentration of plates has been tested both before and after any ethanol conditioning step in an assay and an average concentration determined.

2.12 Materials

2.12.1 Suppliers

Chemicals and salts were obtained from standard suppliers.

The Randox Blood Alcohol Kit was obtained from Randox Laboratories Ltd, County Antrim, UK (BA106).

Embryo dishes were obtained from Raymond A Lamb Ltd East Sussex, UK(E90).

The NAD-ADH Reagent Multiple Test Vials were obtained from Sigma-Aldrich.

The QiaQuick PCR purification kit was obtained from Qiagen.

Taq Expand Long Template PCR systems were obtained from Roche.

Ethanol, analytical reagent grade, was obtained from Fisher Scientific (99.99% ethanol, as measured by gas chromatography).

Sylgard silicone elastomer was obtained from Dow Corning.

Borosilicate glass capillaries GC100-10 were obtained from Harvard apparatus.

2.12.2 Standard buffers

Dents saline
Glucose 1.8g
HEPES 1.19g
NaCl 8.18g
KCl 0.447g
$CaCl_2$ 0.441g
$MgCl_2$ 0.5ml of 1M solution
In 1 litre distilled water
NaOH to pH 7.4

M9
KH_2PO_4 3g
Na_2HPO_4 6g
NaCl 5g
$MgSO_4$ (1M) 1ml
in 1 litre distilled water

Nematode Growth medium (NGM agar)
15g NaCl
12.5g Peptone
75g agar
4875ml dH_2O

Autoclaved, then supplemented with,
5ml sterile cholesterol (5mg/ml in ethanol)
5ml sterile 1M $CaCl_2$
5ml sterile 1M $MgSO_4$
125ml sterile 1M KH_2PO_4

Bleach mixture
5ml Domestos bleach (4.9% $HClO_3$)
5ml dH_2O
10ml 4M NaOH

Glycine buffer
Glycine 3.75g
NaCl 5.84g
NaOH to pH9
in 100ml distilled water

LB
10 g Bacto-tryptone
5 g Bacto-yeast
5 g NaCl
distilled water to 1 litre
pH 7

LB agar
10 g Bacto-tryptone
5 g Bacto-yeast
5 g NaCl
15 g agar
distilled water to 1 litre
pH 7.5

2xYT
Tryptone 16g
Yeast extract 10g
NaCl 5g
In 1 litre distilled water

Loading buffer
5 % bromophenol Blue 250 µl
Glycerol 3 ml
H_2O 7 ml

TBE buffer
54g TRIS
27.5g Boric acid
20ml 0.5M EDTA
pH 8 in 10 litres final

Lysis buffer
2.4g TRIS pH 7.5
14.6g EDTA
11.7g NaCl
0.5% SDS
In 1 litre distilled water

Chapter 3 - Acute Intoxication

3.1 Introduction

The first aim of the study was to characterise the response of *C. elegans* to acute ethanol. For this purpose the response to acute ethanol was defined as being the initial response to the first exposure to ethanol a worm has experienced. Previous work has used a number of behavioural assays based on locomotory behaviour to investigate the dose dependent response to acute ethanol. These are summarised in the Introduction (section 1.11.2) and consistently report that alcohol at concentrations greater than 100mM inhibits locomotion (Davies et al., 2003;Eckenhoff and Yang, 1994;Graham et al., 2008;Kapfhamer et al., 2008;Kwon et al., 2004;Morgan and Sedensky, 1995).

Therefore, as a first step towards identifying ethanol induced behavioural states in *C. elegans*, the locomotory response of wild-type *C. elegans* to acute ethanol exposure was determined over a range of doses based on the previous literature (100-500mM).

3.2 Results

3.2.1 The effect of acute ethanol on movement in liquid (thrashing)

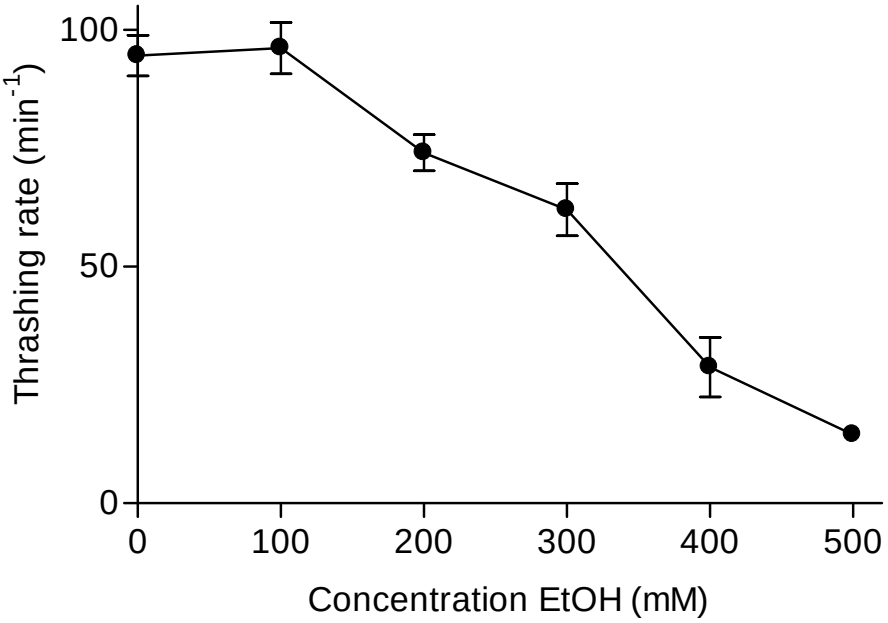


Figure 3.1 Concentration response curve for the effect of acute ethanol on thrashing behaviour. For each concentration, thrashing rate was determined after 20min exposure to ethanol i.e. at steady state (see Figure 3.2). Each point is the mean ±s.e. of at least 9 independent worms.

In liquid *C. elegans* display a characteristic locomotory behaviour known as thrashing (see section 2.5.1). Immersion of *C. elegans* in ethanol (range 100–500mM) inhibited, but did not completely abolish, thrashing behaviour. This effect is concentration-dependent and half-maximal at approximately 300mM (Figure 3.1). The worms were still not completely paralysed at 500mM, which was the highest concentration tested.

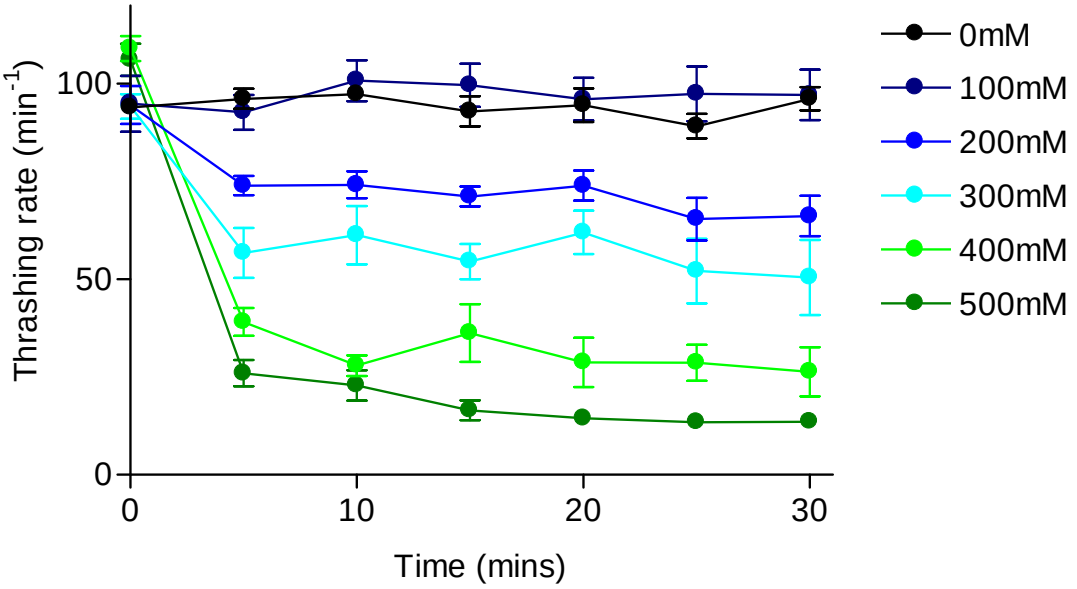


Figure 3.2 Time course for the inhibitory effect of ethanol in the thrashing assay. The worm reaches a steady rate of thrashing before the first time point at 5 min. The zero time point shows the thrashing rate of the worm before the addition of ethanol. Each worm was tested at all time points of one concentration. Results are the mean ±s.e. of at least nine independent worms.

The time course of the inhibitory effect of ethanol on thrashing behaviour was investigated. Notably, at each concentration, the inhibition reached a maximum within 5 min of being added to ethanol (Figure 3.2). After this the level of inhibition was stable for up to 30 minutes.

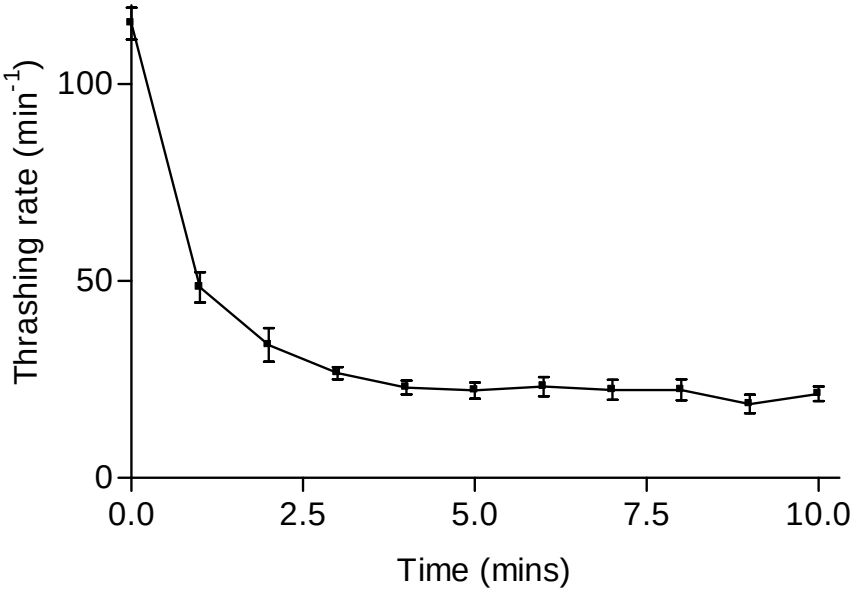


Figure 3.3 Rate of onset of the inhibitory action of ethanol on thrashing behaviour. The thrashing rate was measured at one minute intervals during the first ten minutes of exposure to 500mM ethanol. The zero time point shows the thrashing rate in the absence of ethanol. Each point is the mean ± s.e. of ten independent worms each of which was measured at all time points.

To investigate the onset of the effect of ethanol in more detail, the assay was repeated at a single concentration (500mM) while thrashing rates were measured at 1 min intervals for the first 10 min. The thrashing rate reached a maximum inhibition after a 3 min exposure (Figure 3.3).

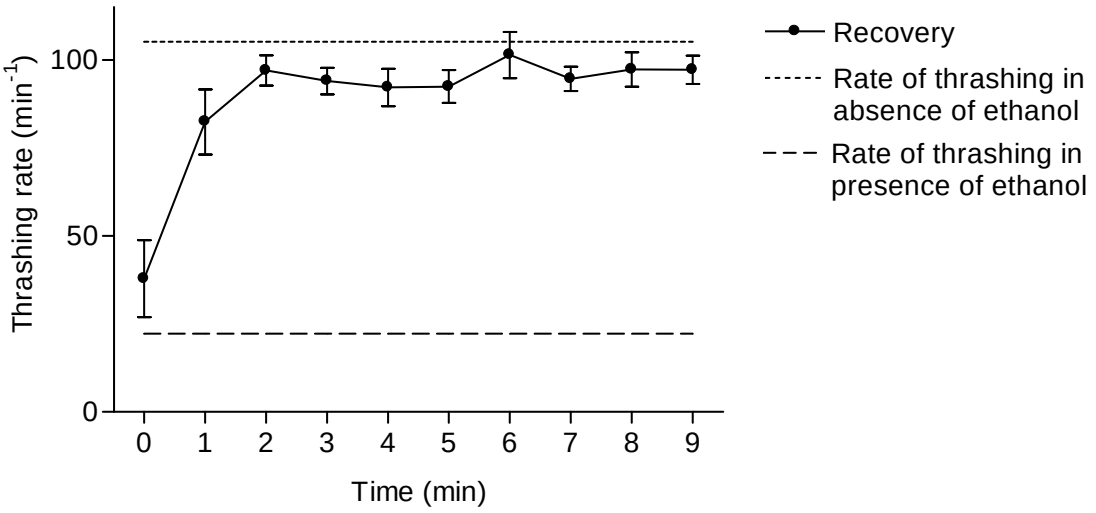


Figure 3.4 Rate of recovery from ethanol. Before the assay the thrashing rate of each animal in Dents saline was recorded. Each worm was then placed in 500mM ethanol for ten minutes and the thrashing rate in ethanol was recorded. The average thrashing rate in the absence and presence of ethanol are shown here in lines across the graph for comparison. At time zero each worm was taken out of ethanol and placed in Dents saline. The rate of thrashing was recorded immediately and every minute afterwards for ten minutes. Each point is the mean ±s.e. for at least nine independent worms each of which was recorded at every time point.

In addition worms that had reached steady state inhibition at 500mM ethanol were removed and placed in ethanol free saline. This allowed the recovery to be measured. There was full recovery and this recovery was complete within 2 minutes (Figure 3.4).

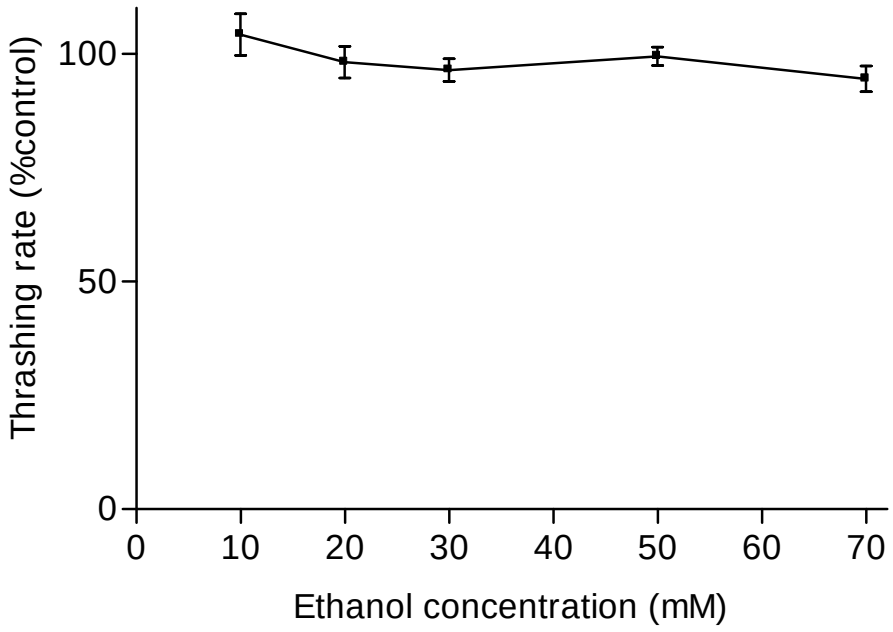


Figure 3.5 Concentration response curve for the effects of 10-70mM ethanol on thrashing behaviour. For each concentration thrashes per min were measured after 20 minutes exposure to ethanol i.e. at steady state. They were then expressed as a percentage of the thrashing rate in the absence of ethanol measured at the same time. Each point is the mean ±s.e. n=10.

Although a threshold for inhibition of >100mM had been observed, lower concentrations of ethanol were tested in the thrashing assay. Concentrations of ethanol in the range 10-70mM had no significant effect ($F_{4,45}$=1.3, P=0.28) on the behaviour of the worm in the thrashing assay (Figure 3.5). This contradicts previous work (Graham et al., 2008) which reported an excitation at 22mM, as described in the introduction (section 1.11.2). This will be discussed in section 3.3.

3.2.2 The effect of acute ethanol on movement on agar

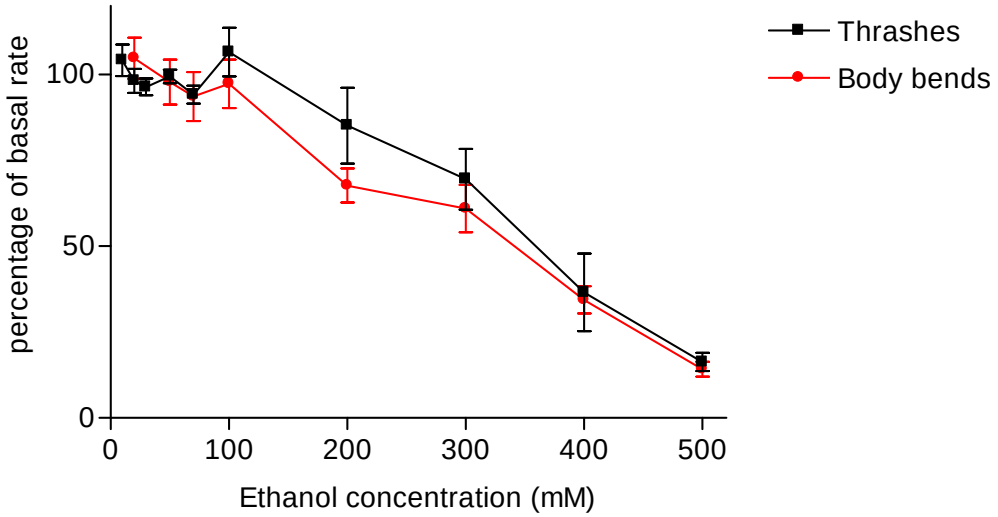


Figure 3.6 Concentration response curve showing frequency of both thrashes and body bends per minute in various concentrations of ethanol as a percentage of their basal frequency in the absence of ethanol. Each point is the mean ± s.e. of at least eight independent worms.

The effect of acute ethanol on movement on agar was investigated. Ethanol containing agar plates were made as described in section 2.3.2. On agar plates worms move with a sinusoidal locomotion. This can be measured by counting body bends. One body bend is defined as the area just behind the pharynx bending in the opposite direction and then returning to its original direction. Acute ethanol in the concentration range 20-500mM inhibited body bends on agar plates to a similar extent as it inhibited thrashing in liquid. Previous work has measured speed on ethanol containing agar plates as mentioned before (Davies et al., 2003). The speed of worm locomotion on plates could be related to the frequency of body bends, or it could be affected by other factors such as the amplitude of body bends. Here the results show that the frequency of body bends on plates is inhibited by ethanol to a similar extent as that reported for speed on plates. They do not provide evidence for hyperactivity in response to low concentrations of ethanol in the body bends assay.

3.2.3 The effect of acute ethanol in the food race

The effect of acute ethanol on behaviour in the food race was investigated. Food race plates were made as described in section 2.3.4. In this assay approximately 50 worms were added to the opposite side of the plate to a point source of *E. coli* OP50 (food) (see Figure 3.7).

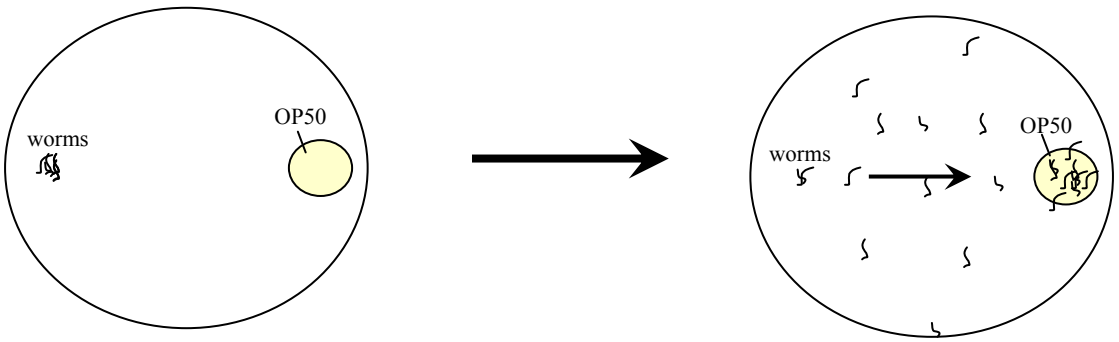


Figure 3.7 Diagram illustrating the food race experiment. Worms are initially plated on the opposite side of a 9cm agar plates to a point source of food. Over time they navigate towards the food. Every ten minutes the number of worms that have reached the food is counted and these worms are removed. Ethanol (when present) has been added to the agar the previous day to give it time to equilibrate (see section 2.11.2).

This assay measures the ability of *C. elegans* to chemotax towards food. Two behaviours have been previously described that may be relevant to the locomotion of *C. elegans* after being placed in the food race. These are the biased random walk seen in *C. elegans* chemotaxis (Pierce-Shimomura et al., 1999) and area restricted search seen when *C. elegans* are removed from food and placed in a food free environment. These behaviours are interrelated as they are both part of *C. elegans* strategy for finding food (Gray et al., 2005). Both of these processes involve variation in the frequency of high angled turns such as reversals and omega turns. In chemotaxis the rate of high angled turns is correlated with the rate of change of attractant concentration over time. In area restricted search it is correlated with the time since removal from food.

In this assay, the worms have been removed from food and directly placed onto food race plates in which a chemoattractant (food) is present on the other side of the plate, so both processes may be relevant to their behaviour.

The rate at which the worms reach the food in this assay is likely to be affected by several different facets of behaviour. It will be affected by the overall speed of worm locomotion, by the frequency of reversals and high angled turns, by the ability of the worm to detect the presence of food and by the ability of the worm to alter its behaviour in response to the detection of food. Ethanol may affect any or all of these facets of behaviour. The food race assay and variations upon it have previously been used to investigate mutants that have altered reversal behaviours (Zheng et al., 2004).

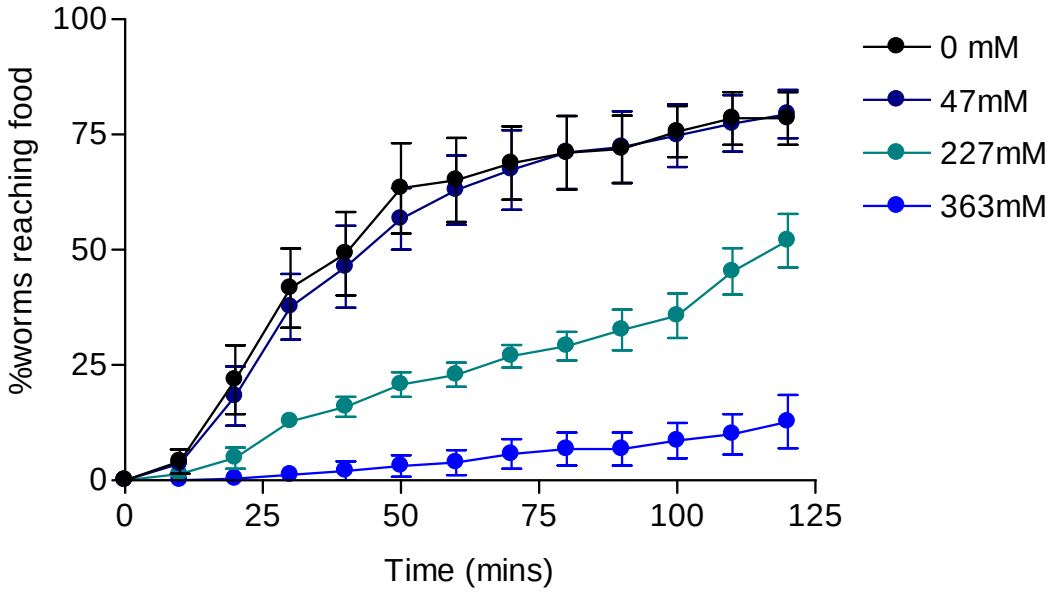


Figure 3.8 Effect of three acute concentrations of ethanol on the percentage of worms reaching the food over a two hour period. Each point is the mean ±s.e. of at least four independent food race assays of approximately 50 worms. The ethanol concentrations of the agar plates were measured subsequent to the experiment.

The food race assay was carried out using plates at 0mM, 47mM 227mM or 363mM ethanol. The plates at higher ethanol concentrations (227mM and 363mM) reduced the ability of worms on them to reach the food. The plates at the lower concentration of ethanol (47mM) had no effect on the ability of worms to reach the food relative to controls (Figure 3.27). This was similar to the effects seen in the other assays where the threshold for the inhibitory effect of ethanol was >100mM.

3.3 Discussion

In the experiments described in section 3.2, acute ethanol inhibited the locomotion of *C. elegans* in the thrashing, body bends and food race assays over a range of external concentrations from 100 to 500mM. This agrees with the published literature described in section 1.11.2 (Davies et al., 2003;Eckenhoff and Yang, 1994;Graham et al., 2008;Kapfhamer et al., 2008).

The ethanol-induced reduction in the ability of the worms to reach the food in the food race (Figure 3.8) is more marked at lower concentrations than that described by Kapfhamer et al. in their dispersal assay. No effect was seen at 200mM ethanol in the dispersal assay, but in the food race assay a clear inhibition is seen at 227mM. In the food race worms are placed at the opposite side of a 9cm plate to a point source of food, and the rate at which they reach the food is recorded. In the dispersal assay worms are placed in the centre of a 10cm plate with food spread all around the edge, and again the rate at which they reach the food is recorded. In both assays ethanol is present in the agar.

There are two main differences between these two assays which could affect the sensitivity with which they detect ethanol-induced inhibition of locomotion. The first is the distance which the worms have to travel. Worms do not have to travel as far in the dispersal assay, a slight impediment to their locomotion might not prevent them reaching the food. However this is unlikely as one would expect the rate at which they reached the food to be altered, even if the proportion that had reached the food at the end of the assay were unchanged.

The second main difference between the assays is that in the food race the food is presented as a point source, so the worms must detect the direction of the food and navigate towards it. In the dispersal assay there is food in all directions, so less navigation is required. It is possible therefore that as well as inhibiting locomotion; ethanol is interfering with the ability of the worms to detect food or to navigate towards it once detected.

One case in which the data shown here does not agree with the published literature is that it does not demonstrate a significant effect of ethanol in the concentration range 0-100mM in the thrashing assay, the body bends assay or the food race assay. This contradicts previously published data (Graham et al., 2008). In this paper transgenic rescues of worms containing a null mutation in the gene *unc-18* (*unc-18 e81*) were made, using either the wild-type *unc-18* gene, or an *unc-18* gene containing a single nucleotide polymorphism D214N. Both rescues used the endogenous *unc-18* promoter. The response of these worms to ethanol in the thrashing assay was measured. The wild type rescue showed similar inhibition by ethanol to that seen in the assays described here (Figure 3.1). The D214N rescue showed reduced inhibition by ethanol compared to the wild type rescue. In addition the wild type rescue showed hyperactivity at 22mM ethanol which was not seen in the D214N rescue. The response of N2 worms to 22mM ethanol was not tested in that study. This study does not show this hyperactivity in the thrashing assay in response to concentrations between 10 and 70mM in wild type worms (Figure 3.5).

The thrashing assays used were very similar so this is unlikely to be the cause of the discrepancy between these results. It is possible that this is due to a difference between the strains, as the N2 used in this study, and the wild type transgenic rescue of the *unc-18* null mutants, have different genetic backgrounds. However the strain containing the *unc-18 e81* allele had been outcrossed with N2, which would be expected to remove background mutations. In addition the transgenic rescue had been performed with the wild type gene and the wild type promoter and multiple transgenic rescues with the wild type gene had been performed and found not to alter the basal locomotory rate. This would argue against any change in the expression level of *unc-18* between the transgenic rescue and the wild type N2, however it is still possible that this is an effect of *unc-18* overexpression.

Further investigation will be required to determine the response of *C. elegans* to low concentrations of ethanol. In this context it is interesting to note that two papers describe *C. elegans* as briefly increasing their locomotion in response to initial exposure to high concentrations of ethanol before becoming inhibited. This could be a response to low concentrations of ethanol before the final concentration of ethanol inside the worms was reached. The worms were described as being fully inhibited within ten minutes (Kwon et al., 2004;Morgan and Sedensky, 1995).

Steady state thrashing rate had previously been described as being reached in less than five minutes (Morgan and Sedensky, 1995), with recovery in less than ten minutes (Kwon et al., 2004). This has been investigated further here and it has been shown that worms reached a steady rate of thrashing within 3 minutes of immersion in 500mM ethanol and recovered within 2 minutes of removal from this ethanol solution.

This indicates that the ethanol concentration inside the worm rapidly reaches a steady concentration.

In conclusion the response to acute ethanol is a well established paradigm in *C. elegans*. The results in this chapter largely agree with the published work and extend it by reporting the rapid kinetics of the onset of and recovery from ethanol's effects in intact animals. This enables the use of these assays to investigate the effects of chronic ethanol on *C. elegans*.

Finally it is interesting to note that the acute ethanol concentrations at which *C. elegans* show inhibition of locomotion (>100mM) in the assays here and in the previously published work, are in fact greater than the blood-alcohol concentrations that would kill a human (approx 87mM) (Koob and Le Moal, 2006). This will be discussed further in Chapter 4.

Chapter 4 - The internal ethanol concentration of *C. elegans*

4.1 Introduction

The concentration range over which ethanol exerts effects on *C. elegans* (see Chapter 3) is much higher than that required to exert an effect on the mammalian brain. Human responses to acute ethanol were described in the Introduction (section 1.4)

The data therefore show a great discrepancy between the dose of ethanol at which *C. elegans* and humans will show a given level of inhibition. *C. elegans* show no inhibition at all in the assays described in Chapter 3 at concentrations greater than those that would kill most people (see Table 1.1).

An explanation that has been advanced for this discrepancy is that the *C. elegans* cuticle has a very low permeability to some exogenous chemicals (Davies et al., 2003). Thus the lipophilicity of drugs has a strong bearing on the concentration that is achieved in target tissues following external application. It is not uncommon for polar drugs to be applied at a concentration 1000 fold higher than their predicted affinity for the target (Holden-Dye and Walker, 2007). This is the reason that many drugs show large discrepancies between concentrations required to produce an effect on living *C. elegans* and the concentrations effective on mammalian cells (Rand and Johnson, 1995). Ethanol is a very small non-polar molecule compared to the drugs in question; nevertheless it has been proposed that the ethanol concentration inside *C. elegans* is likely to be very much lower than the medium due to a presumed low permeability to exogenous chemicals (Davies et al., 2003). This supposition was supported by measurements of the ethanol concentration inside the worms which estimated a

concentration of approximately 22mM at an external concentration of 400mM, and 29mM at an external concentration of 500mM (Davies et al., 2003).

However, the current work has shown that the rate of thrashing in ethanol reaches a steady state inhibition within 3 minutes and this is completely reversed within 2 minutes of removal from ethanol (Figures 3.3 and 3.4). The most parsimonious explanation for this behavioural observation is that the internal concentration has reached equilibrium in this time frame. There are two possible routes of entry of ethanol into the worm: by ingestion through the mouth and/or directly across the cuticle. Therefore, there are two possible explanations for the steady-state effect on thrashing following immersion in ethanol. If the route of entry is primarily by ingestion, then the steady-state effect of ethanol will be attained when the rate of absorption equals the rate of elimination (by metabolism and/or excretion). Alcohol dehydrogenase activity has been identified in *C. elegans* (Williamson et al., 1991) so a high rate of metabolism is a possible explanation for a low concentration of ethanol in *C. elegans*.

However experiments performed using the pharyngeal pumping assay provide a contradiction to the idea that the internal concentration of ethanol in the worm is much lower than that in the surrounding medium. Pharyngeal pumping is an established bioassay for neuroactive compounds (Avery and Horvitz, 1990) and can be performed on intact animals in which the cuticle will present a barrier to the access of drugs to the pharynx or on a dissected semi-intact preparation of the anterior region of the worm that contains the muscle and the pharyngeal neural circuit in which the pharynx will be exposed to the surrounding saline. The concentration-dependence of

the effect of ethanol on the pharynx has been shown to be very similar in both preparations, although the onset and offset was slower in the intact preparation (Mitchell et al., 2007) (see Figures 4.1 and 4.2).

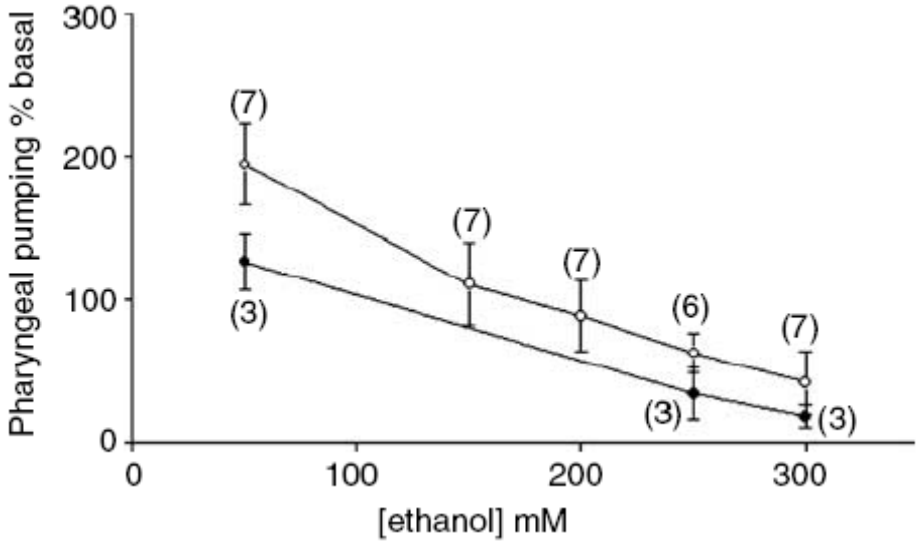


Figure 4.1: A comparison of the concentration-dependence of the effect of ethanol on pharyngeal pumping in intact animals (filled circles) and exposed pharynxes (open circles). The effect of ethanol is expressed as the % pumping rate of the pharynx compared to basal pumping rate, that is, before the addition of ethanol. Each point is the mean ± s.e. of n determinations. (from (Mitchell et al., 2007))

It is also of note that the concentration-dependence (>100mM ethanol for paralytic activity), and the time course (approximately 3–5 min), of the inhibitory effect of ethanol on the pharynx were similar to that for the thrashing behaviour. As the concentration-dependence of the inhibitory effect of ethanol on the pharynx is similar whether it is applied externally or internally, this supports the contention that the concentration of ethanol inside the worm at steady-state is most likely to be very near to the external concentration. If the ethanol concentration inside the worm were 22mM when 400mM was applied externally, one would expect 22mM to have a

similar effect on a cut head to that which 400mM did on the intact preparation.

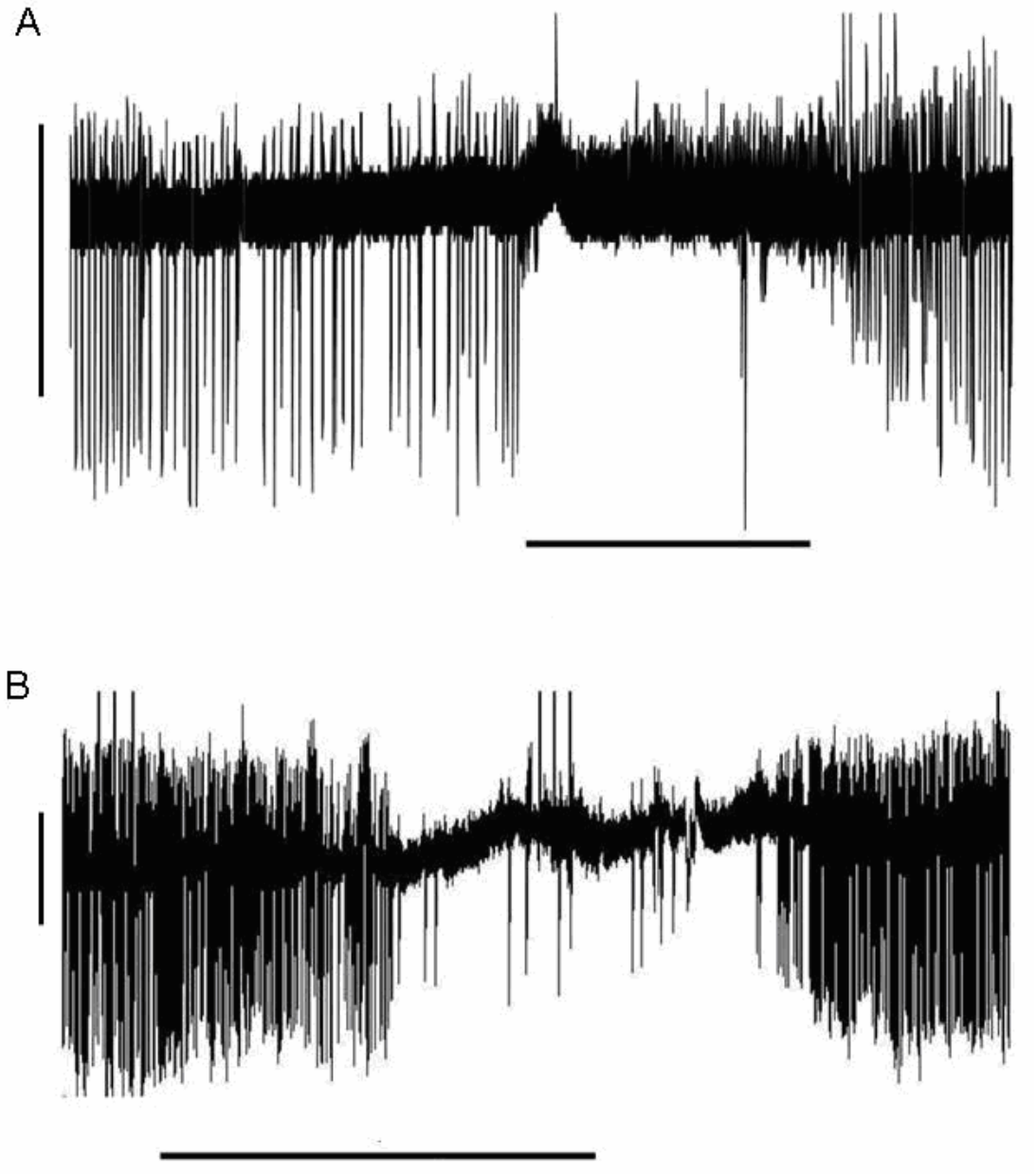
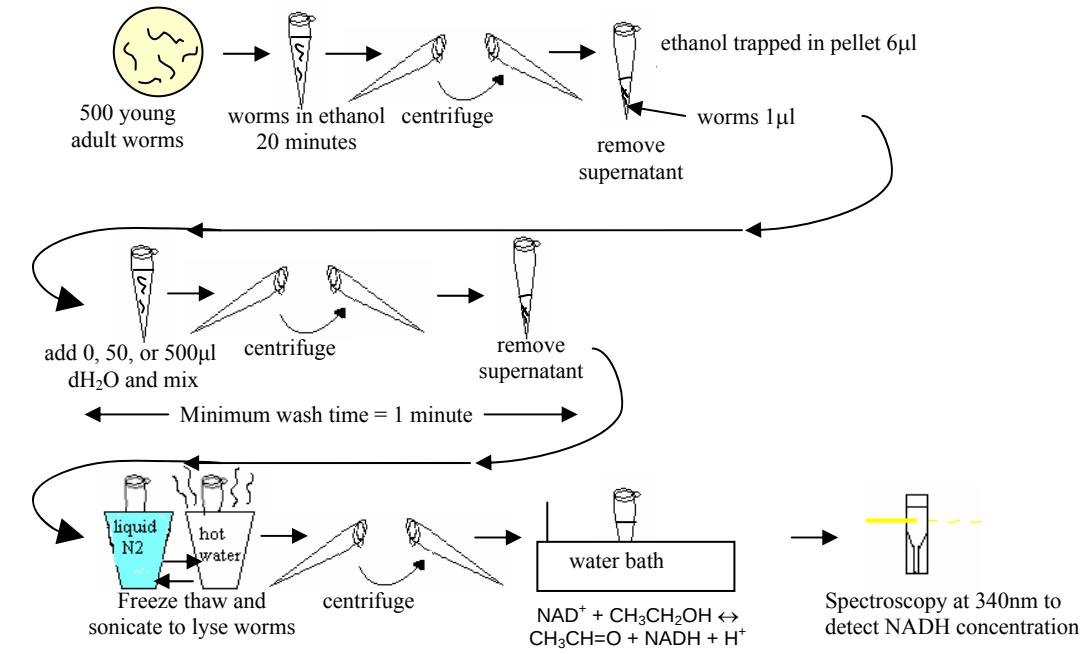


Figure 4.2 Recordings of pharyngeal activity (EPG) in exposed (A) and intact (B) worms. In (B) the cuticle of the worm is intact. The pharynx consists of radial muscle, which rhythmically pumps to maintain the feeding activity of the animal. Each vertical line represents the electrical activity associated with a single muscle pump; therefore this provides a read-out of the activity of the muscle. Each trace shows 10 min of recording and an example of the inhibitory effect observed with 250mM ethanol. Ethanol was added and removed from the recording chamber by replacing the solution with a pipette. The duration of application of ethanol is indicated by the bar. The vertical scale bar is 1mV. Note that the onset and offset of the response to ethanol in (B) is slower than in (A), but the level of inhibition is very similar. 5-HT was included in both experiments to stimulate a basal rate of pumping against which inhibition could be measured. In (A) this was 50 nM and in (B) 1mM (the cuticle is not very permeable to 5-HT hence the higher concentration required in the intact preparation). (from (Mitchell et al., 2007))

To investigate the apparent contradiction between these results showing that the cuticle did not provide a barrier to the effects of ethanol and the previously published work describing a very low internal ethanol concentration in *C. elegans*, the accuracy of the biochemical assay for estimating internal ethanol concentration was investigated.

4.2 Results

A



B

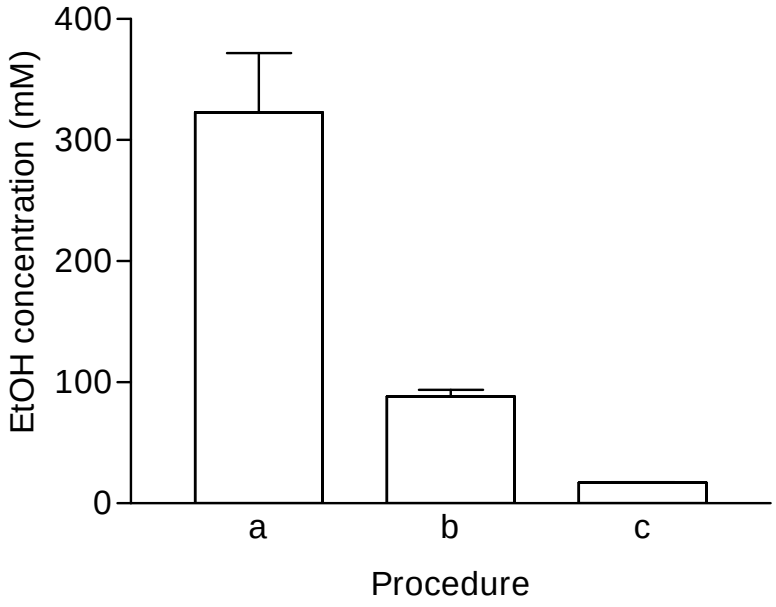


Figure 4.3 Estimation of the internal ethanol concentration following exposure to 500mM ethanol for 20 min. A. Cartoon of method used to estimate ethanol concentration in the worms. B. Estimated ethanol concentrations obtained (a) measurement from animals that were exposed to ethanol but not washed; (b) and (c) measurements from animals exposed to ethanol and subjected to different wash steps: (b) 50µl water; (c) 500µl water. Values are the mean ±s.e. of 6, 9 and 3 assays respectively. The protocol employed was adapted from the published method (see section 2.2) that has been used by others (Davies et al., 2003) to estimate the internal ethanol concentration of the worms following exposure to 500mM ethanol. The estimate of obtained was 17 ±0.5mM (n=3) and close to the published values (e.g. 29mM) (Davies et al., 2003). This is shown as procedure (c).

However, the procedure used to obtain this estimate requires that the animals be washed in cold buffer before the measurement (see Figure 4.3A). The observation that animals exposed to ethanol fully recover from the inhibition of motility within 2 min (Figure 3.4) suggests that a significant amount of ethanol may be lost from the inside of the animals during the protocol. Indeed, because the behavioural effects of ethanol reversed so rapidly, the possibility that the ethanol assay in fact measures residual ethanol in the worm pellet following centrifugation was considered. An approximate estimate of the volume occupied by 500 worms (2nl/worm) indicated that this volume could be as low as 1µl (Knight et al., 2002), whereas the estimated volume of the worm pellet overlaid with ethanol was ≥5µl. The volume of the worm pellet was estimated by eye relative to a range of comparison tubes. 5µl is the minimum estimate. Therefore in the worm pellet the ethanol is in excess and the dilution of this ethanol could be all that the assay is measuring.

This was tested directly in a further series of experiments in which the influence of wash volume during the assay procedure on the estimate of internal ethanol concentration was determined. The data shown in Figure 4.3B indicate that the estimate of internal ethanol concentration increases as the volume of the wash buffer decreases. As a further confirmation of this the effect of wash time on the measured ethanol concentration was tested. An increased time in the wash step did not significantly affect the concentration of ethanol measured (Figure 4.4). The wash time used in Figure 4.3 was the fastest wash time possible in our hands.

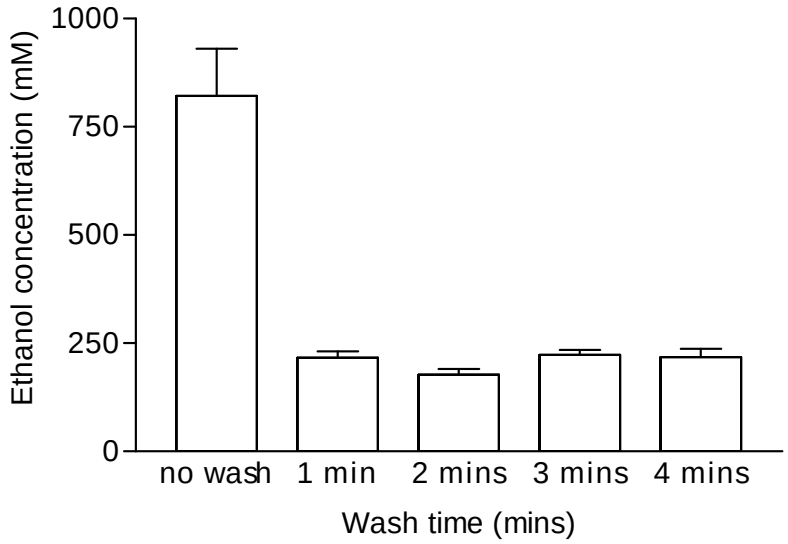


Figure 4.4 Estimation of the internal ethanol concentration following exposure to 1M ethanol for 20 min. The columns indicate the estimated ethanol concentration obtained: (no wash) measurement from animals that were exposed to ethanol but were not washed; (1, 2, 3, and 4 min) measurement from animals exposed to ethanol and subjected to wash steps of different times in 50µl water. Values are the mean ±s.e. of 5, 6, 8, 2 and 6 assays respectively.

This further indicates that the assay is in fact only measuring residual ethanol in the worm pellet following centrifugation as, if the internal worm ethanol is lost to the wash, then increasing the wash time should further reduce the internal ethanol concentration unless the ethanol is lost very rapidly, but if the wash is simply removing contaminating external ethanol surrounding the worm then this effect should happen immediately and not be time dependent. If the internal worm ethanol is lost very rapidly this would be another indication that the internal worm ethanol concentration would be likely to equilibrate with the external ethanol concentration.

In summary the concentration of ethanol measured by these assays is dependent on the volume of distilled water that the pellet, which contains ethanol and ethanol containing worms, is washed in. In fact all of these results are consistent with the dilution of a 10-18µl drop of the initial concentration of ethanol being diluted by the appropriate wash volume. This assay is therefore not providing an accurate measure of ethanol concentration in the worm.

4.3 Discussion

These results show that the previously used biochemical assay for the measurement of ethanol concentration does not provide a credible estimate of internal ethanol concentration. Indeed, the assay appears to measure the concentration of the ethanol surrounding the worm pellet, which is diluted as expected during the wash step.

It has previously been shown that the onset of and recovery from ethanol intoxication in the worm is extremely rapid when measuring thrashing in liquid (Figures 3.3 and 3.4), suggesting that ethanol is likely to rapidly equilibrate across the water-permeable cuticle of the worm.

It has also been shown that ethanol can affect the rate of pharyngeal pumping in a concentration dependent manner that is unaffected by the presence or absence of the cuticle (Mitchell et al., 2007) (see Figures 4.1 and 4.2). Taken together, these results indicate that the *C. elegans* cuticle does not seem to be a significant diffusion barrier for ethanol when measuring behavioural consequences of ethanol exposure.

C. elegans have been shown to contain alcohol dehydrogenase (Williamson et al., 1991) and thus probably metabolise ethanol to some extent. However the fact that direct exposure to the bath solution does not significantly alter the extent of inhibition of pharyngeal pumping by ethanol indicates that any metabolism of ethanol that occurs does not greatly alter the internal concentration. If the cuticle does not provide a significant diffusion barrier to ethanol, the internal concentration is likely to remain clamped by the external reservoir. It is likely that the internal ethanol concentration of the worm is thus similar to the bath solution.

One piece of evidence which seems to contradict the statement that the internal ethanol concentration of the worm is similar to the bath solution is the fact that ethanol causes an increase in the SLO-1 dependent current in *C. elegans* CEP neurons *in situ* at doses similar to those in which it acts in mammalian cells. 20mM ethanol caused a 20±4% increase and 100mM ethanol caused a 29±5% increase in the SLO-1 dependent current (Davies et al., 2003). This was shown to be caused by an increased frequency of channel opening; P_{open} increased from 0.098 in the control to 0.169 at 100mM ethanol (172% of control).

SLO-1 is a homolog of the pore-forming α subunit of the mammalian BK channel. Ethanol has been shown to increase the open probability of BK channels in isolated rat neurohypophysial terminals in concentrations between 10-100mM to up to 450% of controls (Dopico et al., 1996), the potentiation observed at 10mM corresponding to approximately 150% of control values (or a 50% increase).

It is possible that at higher concentrations than 100mM, ethanol would cause a greater increase in the SLO-1 dependent current in *C. elegans* which would explain the reduction in locomotion at concentrations between 100-500mM, which has been shown to be *slo-1* dependent (Davies et al., 2003). The 20-29% increase in this current between 20-100mM could contribute, along with other effectors, to the potentially more subtle effects of ethanol at these lower concentrations.

It is interesting to note that the *slo-1* gain of function mutants (*ky389gf* and *ky399gf*), which show behavioural depression similar to ethanol treatment but not immobility,

show an increase in the SLO-1 dependent current from the same neurons of 54% and 60% respectively (Davies et al., 2003). This is more than twice the effect of 100mM ethanol applied internally and may thus be more similar to the effect of 200mM or more ethanol, which would be expected to cause behavioural depression.

Why is there such a great discrepancy between the response of humans and *C. elegans* to the same concentrations of ethanol? The vast difference between the LD50 value for humans (87mM (Koob and Le Moal, 2006)) and *C. elegans* (1890mM (Dhawan et al., 1999)) could be due in part to that fact that, as *C. elegans* do not require rhythmic muscular movements to exchange oxygen with the environment, they can survive paralysis and severe locomotory impairment, which humans cannot. Alcohol poisoning in humans leads to respiratory depression which causes death. Therefore concentrations that severely inhibit rhythmic behaviours in *C. elegans* (300mM and higher) are clearly likely to be fatal for humans. Acute alcohol poisoning is not necessarily an effect of neurotoxicity, and some studies have even shown that acute ethanol at intoxicating concentrations (approx 54mM) can have neuroprotective effects in mammalian systems (Farber et al., 2004).

At concentrations of ethanol that would be relevant to intoxicating doses in humans (<50mM), you would expect to see much more subtle effects in the worm. An example of such effects would be the increase in the SLO-1 dependent current described by Davies et al. (Davies et al., 2003). Another example would be the hyperactivity in the thrashing rate shown by Graham et al. (Graham et al., 2008).

However, the doses at which inhibition of locomotion starts to be seen in the worm (>100mM) are still slightly higher than the LD50 in humans (87mM). It is possible that *C. elegans* may have evolved to endure environments where they often encountered high levels of ethanol, such as rotting fruit. It has been reported that *C. elegans* are often found in such environments (Felix, 2007). In which case, some of their proteins might have a response to ethanol in which the dose response curve is shifted to the right in comparison to the human homologs. This would explain why *C. elegans* show subtle intoxicating effects at 10-100mM and more sedative effects at 100-300mM, as opposed to intoxicating doses of 10-40mM and sedative ones of 40-90mM in humans. It was, in fact, suggested by Morgan and Sedensky in 1995 that the resistance of *C. elegans* to all volatile anaesthetics may have developed as a selective advantage, due to the free-living nematode's normal surroundings and relative permeability to simple organic compounds (Morgan and Sedensky, 1995).

However, despite this, *C. elegans* can still be considered to be a good model for the effects of ethanol on humans as they show the qualitatively similar response of possible hyperactivity at low doses, followed by sedation at higher doses.

It is interesting to note that there is also controversy surrounding internal ethanol concentration in *Drosophila*, with estimates for the ethanol concentration in the fly following a sedative dose of ethanol ranging from 15mM (Berger et al., 2004) to 235mM (Cowmeadow et al., 2005) ethanol. Nevertheless *Drosophila* has been used extensively to investigate the mechanisms of ethanol intoxication and tolerance.

In summary, to understand *C. elegans* as a relevant model for human ethanol intoxication, it is necessary to define the concentration dependence of the behavioural response in the worm. Evidence is provided here that the *C. elegans* cuticle does not seem to be a significant diffusion barrier for ethanol when measuring the behavioural consequences of ethanol exposure and thus the external concentration approximates to the concentration relevant to the neuroactive properties of ethanol in these assays. Accordingly it is recommended that future studies aim to investigate the responses of *C. elegans* to concentrations of ethanol low enough not to cause a total reduction in locomotion. Later sections of this study use concentrations that cause at most a 50% reduction in locomotion. These experiments enable one to better understand the relevance of experiments conducted in *C. elegans* to effects seen in humans.

Chapter 5 - The effect of chronic exposure to ethanol on *C. elegans*

5.1 Introduction

This chapter describes investigations into the chronic effects of ethanol on *C. elegans*. In Chapter 3 it was demonstrated that *C. elegans* undergoes intoxication in response to acute ethanol. This intoxication is characterized by a reduction in the thrashing rate in liquid, the rate of body bends on agar and the ability to reach the food source in the food race assay. It was shown that this is a dose dependent effect over a range 100-500mM which is half maximal at approximately 300mM.

When a worm is initially placed in ethanol or on an ethanol containing agar plate it reaches a steady level of inhibition in less than five minutes. This change in behaviour is completely reversible after a two minute wash in saline solution to remove residual ethanol. This is the worm's response to an acute exposure to ethanol. If the worm is left on ethanol for an extended period of time its behaviour may change over time. This could then affect its subsequent behaviour both on and off ethanol even after removal of residual ethanol. This is the worm's response to a chronic exposure to ethanol. Chronically exposing worms to ethanol can also be described as conditioning them with ethanol. A worm that has never been exposed to ethanol before is described as naive. A worm that has been chronically exposed to ethanol is described as conditioned.

The chronic effects of ethanol on *C. elegans* were investigated so that *C. elegans* could be used as a model for aspects of alcohol dependence. As was described in the introduction not all aspects of alcohol dependence in humans are capable of being modelled using *C. elegans*. However the development of drug dependence requires

homeostatic neuroadaptation to the continuous or repeated presence of the drug, in this case ethanol. This has also been described in the introduction but, to summarise briefly, neural circuits which are activated by ethanol appear to be downregulated during chronic ethanol exposure and vice versa. This leads to tolerance to the effects of ethanol. Some of these changes will persist if ethanol is removed leading to withdrawal symptoms (Koob and Le Moal, 2006).

This chapter describes the development of paradigms to model tolerance and withdrawal after chronic exposure to ethanol in *C. elegans*. Some chronic effects of ethanol have been previously described in *C. elegans*. These were discussed in the Introduction (sections 1.11.3 and 1.11.4). In this study tolerance will be defined as a reduction in the effect of intoxicating concentrations of ethanol on the worm after chronic exposure to ethanol. Thus if a conditioned worm and a naive worm are observed when exposed to the same intoxicating concentration of ethanol, and the conditioned worms shows significantly less of a response, the conditioned worm will be considered to be tolerant.

Withdrawal is defined as a change in behaviour of a worm in the absence of ethanol after chronic exposure to ethanol. Thus, if a conditioned worm and a naive worm are observed in the absence of ethanol and the conditioned worm behaves significant differently to the naive worm, it may be showing withdrawal. However chronic exposure to a harmful substance such as ethanol could change behaviour in more than one way (see Introduction section 1.11.5). If the worm has undergone neuroadaptation to the presence of ethanol, and is therefore undergoing withdrawal in the absence of ethanol, it would be expected that ethanol could rescue this effect. This is called relief

from withdrawal in this study. Thus if a conditioned worm and a naive worm are observed in the absence of ethanol and the conditioned worm behaves significant differently to the naive worm, and this effect can be at least partially relieved by a low concentration of ethanol, the worm will be considered to be withdrawn.

This chapter describes the effect of exposing *C. elegans* to six main conditions.

*Conditioning***Test*	**No ethanol**	**Low ethanol**	**High ethanol**
No conditioning (naive to ethanol)	Control 	Naive low 	Naive high/ Intoxication 
Conditioning	Withdrawal 	Relief 	Tolerance 

For these purposes high ethanol was defined as approximately 250-350mM ethanol, a concentration range that causes an approximately half-maximal intoxication response when applied acutely (see Figures 3.1 and 3.6) and low ethanol is defined as 40-90mM ethanol, a concentration range that was observed not to produce a response on locomotion when applied acutely in our hands (see Figures 3.5 and 3.6).

In this chapter the food race assay was used to investigate changes in the behaviour of *C. elegans* in response to chronic ethanol (see section 2.5.5 for method). This assay is a model of the ability of *C. elegans* to move towards food. Two behaviours have been described that may be relevant to the locomotion of *C. elegans* after being placed in the food race. These are the biased random walk seen in *C. elegans* chemotaxis (Pierce-Shimomura et al., 1999) and area restricted search seen when *C. elegans* are removed from food and placed in a food free environment. These behaviours are interrelated as they are both part of *C. elegans* strategy for finding food (Gray et al., 2005).

Pierce-Shimomura et al. described chemotaxis to a point source in worms that had previously been off-food for 0.5-2hrs. They showed that each worm spent periods of time moving in a single direction (runs) interrupted by periods of time turning (pirouettes). These pirouettes include reversals followed by changes of direction, reversals followed by omega turns and unaccompanied omega turns. The frequency of pirouettes was correlated with the rate of change of attractant concentration over time, but not with the absolute concentration of attractant. Thus when worms were moving towards the attractant they would tend to perform less reversals than when moving away from the attractant.

Gray et al. describe the behaviour of a worm when it has been initially removed from food which has been described as area restricted search. On food worms move forward slowly and perform frequent, short reversals followed by low angled turns. This behaviour keeps them from moving very far. When initially removed from food (first 12 minutes) they enter a local search state characterised by a high frequency of long reversals and omega turns and a lower but still reasonably high frequency of short reversals. After a longer period (35-40 minutes after removal from food) they enter a dispersal state associated with infrequent reversals and omega turns. The local search state is not reset by touch, only by food (Hills et al., 2004), so this process would not have been occurring in the chemotaxis experiments described earlier (Pierce-Shimomura et al., 1999) as after 0.5-2 hours off food the worms would have entered the dispersal state and thus local search behaviour would not confound the chemotaxis behaviour when worms were on placed on the chemotaxis place.

In the food race assay worms have been removed from food and directly placed onto food race plates in which an attractant (food) is present, but far away from the worms so both processes may be relevant to their behaviour. The rate at which the worms reach the food in this assay is therefore likely to be affected by several different facets of behaviour. It will be affected by the overall speed of worm locomotion, by the frequency of reversals and high angled turns, by the ability of the worm to detect the presence of food and by the ability of the worm to alter its behaviour in response to the detection of food. Ethanol may affect any or all of these facets of behaviour.

If the development of neuroadaptation to ethanol in the food race can be demonstrated further investigations will consider if this is due to effects on reversals, omega turns or the overall speed of locomotion. They will also investigate if ethanol affects the alteration in the behaviour of a worm over time, when placed on a food race plate.

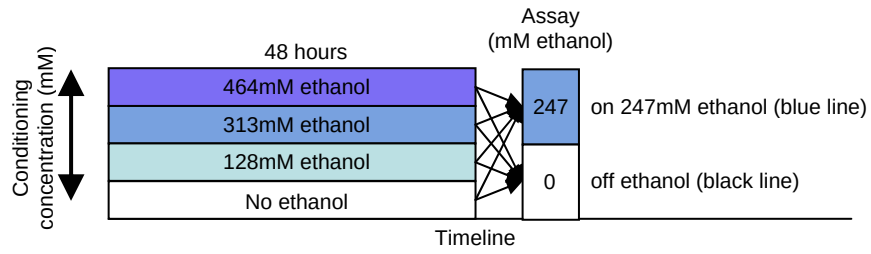
In conclusion, in order to model aspects of alcohol dependence in *C. elegans* both intoxication, which can be improved by tolerance, and withdrawal, which can be partially relieved by a low dose of ethanol, needs to be demonstrated. This will indicate that *C. elegans* are undergoing neuroadaptation to ethanol. If these conditions are met the aim will be to investigate in more detail which behaviours are affected by this neuroadaptation in order to further characterize how ethanol is exerting its effects on *C. elegans*.

5.2 Results

5.2.1 Chronic exposure to ethanol does not produce a definite trend towards tolerance or withdrawal measured in the body bends assay

In order to investigate the effects of long term exposure to ethanol in *C. elegans* the body bends assay was initially used (see section 2.5.2). Worms were exposed to one of three concentrations of ethanol for 48 hours. They were then washed to remove all residual ethanol (see section 2.11.1). The rate of body bends on non-food plates was measured, both on ethanol (to see if the worms had become tolerant) and off ethanol (to see if the worms were withdrawn).

A



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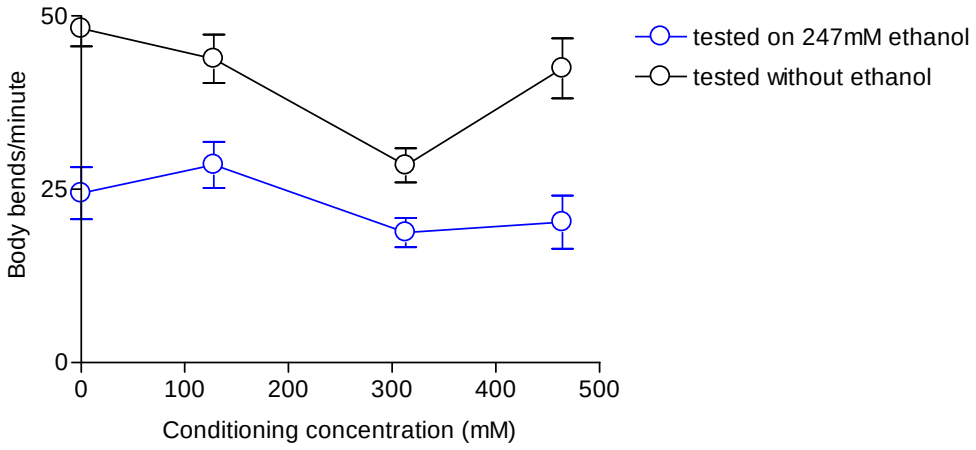


Figure 5.1 Effect of 48 hours exposure to various concentrations of ethanol on body bends on or off ethanol. (A) Timeline of the experiment. (B) On this graph the x axis shows the concentration of ethanol at which the worms were incubated for 48 hours. The black line shows the subsequent rate of body bends in the absence of ethanol. The blue line shows the rate of body bends in the presence of 247mM ethanol.

If *C. elegans* demonstrated neuroadaptation to ethanol using this assay it would be expected that worms which had been conditioned on ethanol would show less of a decrease in locomotion in response to acute ethanol than control worms i.e. tolerance (blue line). Potentially, a change in locomotion when removed from ethanol would also be expected i.e. withdrawal (black line).

48 hours exposure to 247mM didn't produce a definite trend towards either a tolerance or a withdrawal effect (Figure 5.1). However there is a significant difference between the behaviour of the worms tested without ethanol that have been conditioned at the mid-range 313mM concentration and the controls ($P<0.0001$ $t_{26}=5.517$).

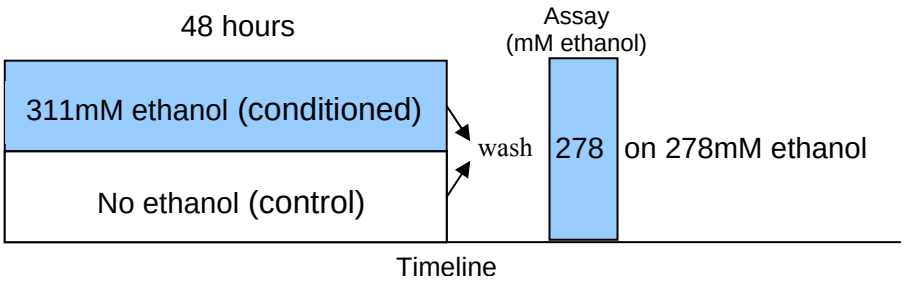
One observation from this was that the worms exposed to the highest ethanol concentration (464mM) appeared to be smaller than the controls. This might have been caused by ethanol interfering with their growth or their osmotic balance. This might be causing additional effects on locomotion which would mask a withdrawal effect. Conditioning concentrations closer to the mid-range concentration (313mM) were used in subsequent experiments.

5.2.2 *C. elegans* show tolerance to ethanol in the food race assay

It was considered that in order to see the response of the worm to chronic ethanol exposure more clearly, it would be necessary to use a test which could investigate a greater range of behaviours which might be affected by ethanol. The effect of ethanol in the food race assay provides a tractable way to extract a quantitative measure of alterations in speed, navigation and chemosensory ability as described in section 5.1.

As shown in Chapter 3 control animals navigate towards the food in a coordinated fashion such that within 2 hours approximately 80% of the animals arrive at the food source. Acute exposure to ethanol (>200mM) during the assay significantly impairs the ability of the animals to reach the food.

A



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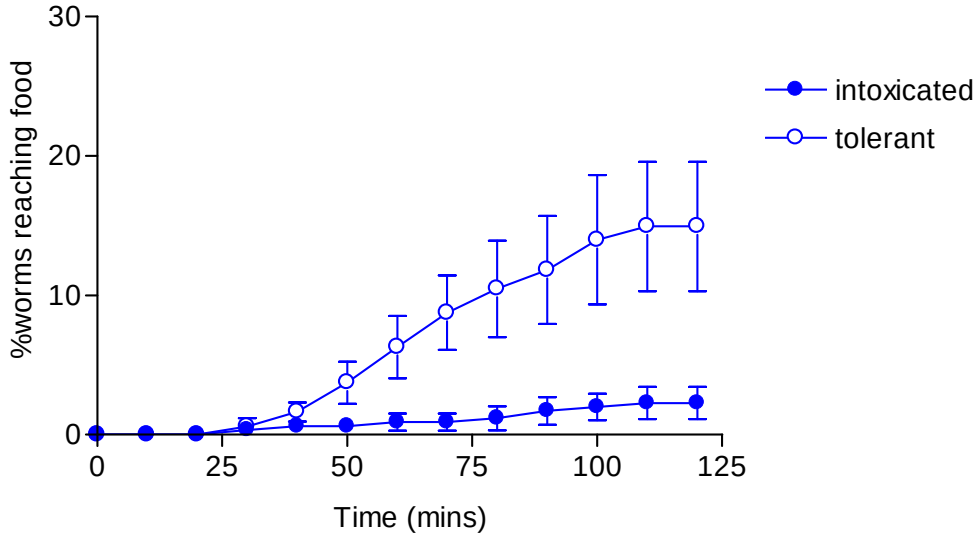

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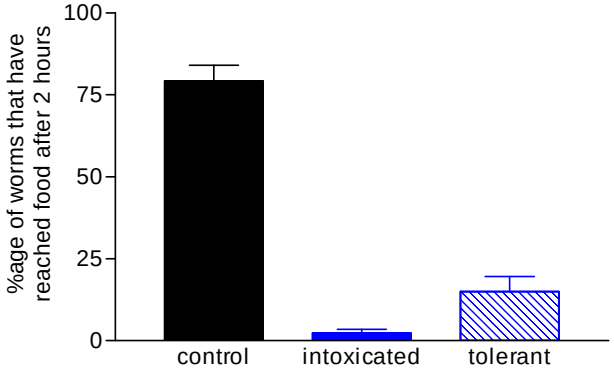


Figure 5.2 Worms conditioned on 311mM ethanol develop tolerance to its effects. (A) Timeline of tolerance experiment, (B) The cumulative percentage of worms reaching the food every ten minutes on 278mM ethanol food race plates. Intoxicated worms have been exposed to ethanol for the first time in this food race and are thus ethanol naive. Tolerant worms have been exposed to 311mM ethanol for 48 hours before the food race. (C) Bar chart showing the percentage of worms that have reached the food after two hours. n=8.

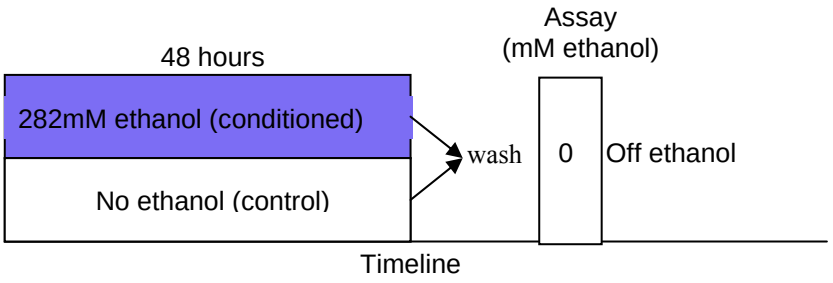
To investigate long term exposure to ethanol using this assay, worms were exposed to one conditioning concentration of ethanol for 48 hours, and then their performance in the food race compared to ethanol naive animals was tested. Worms were conditioned at concentrations in the range 250-350mM ethanol a concentration range that causes an approximately half maximal intoxication response when applied acutely (see Figures 3.1 and 3.6).

At 278mM ethanol worms which have been previously exposed to ethanol perform better in the food race than ethanol naive worms (Figure 5.2). Thus they exhibit ethanol tolerance. A t-test comparing the percentage of worms reaching the food after two hours for the intoxicated and the tolerant worms showed a significant difference (t_{14}=2.641, P=0.0194).

5.2.3 *C. elegans* show withdrawal from ethanol in the food race assay

When conditioned *C. elegans* were tested in the food race in the absence of ethanol they performed very poorly with only 20% reaching the food in 2 hours (Figure 5.3) compared to approximately 80% of the naive control worms. In section 5.1, withdrawal was defined as a change in behaviour of a worm in the absence of ethanol after chronic exposure to ethanol. Thus, if a conditioned worm and a naive worm are observed in the absence of ethanol and the conditioned worm behaves significant differently to the naive worm, it could be described as withdrawal. By this definition *C. elegans* are showing withdrawal in Figure 5.3. However in order to demonstrate that this is an effect of neuroadaptation to ethanol, this response must be able to be relieved by ethanol (see section 1.11.5).

A



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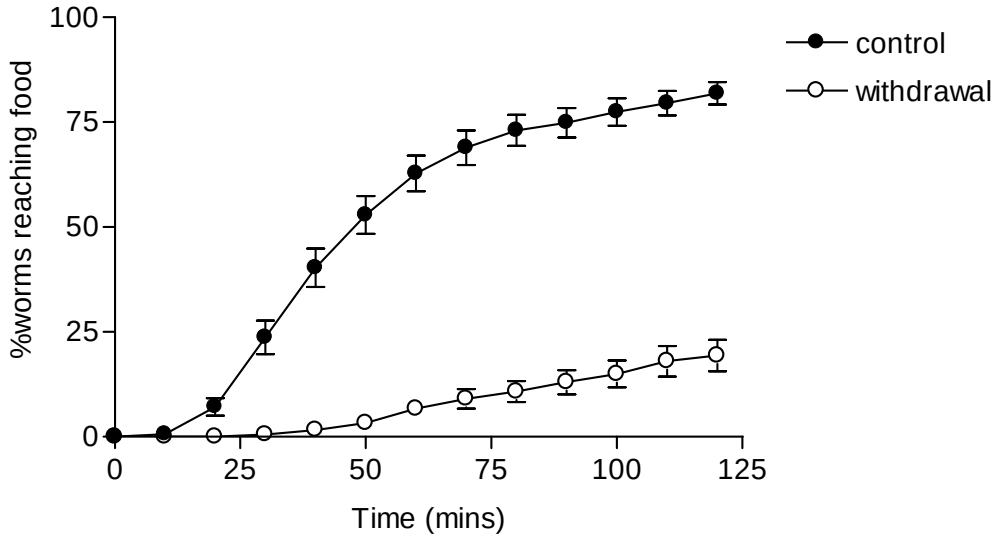


Figure 5.3 Worms conditioned at 282mM ethanol develop withdrawal when removed from ethanol. n=22 (A) Timeline of the withdrawal experiment. (B) The cumulative percentage of worms reaching the food every ten minutes on 0mM ethanol food race plates. Control worms have never been exposed to ethanol. Withdrawn worms have been exposed to 282mM ethanol for 48 hours before the food race.

5.2.4 *C. elegans* shows relief from withdrawal in the food race assay

As discussed in section 5.1, if a withdrawal phenomenon was caused by a homeostatic adaptation to the presence of ethanol then a low dose of ethanol is likely to be able to relieve this behaviour by restoring the balance of neural signalling in the affected networks. This was described as relief from withdrawal.

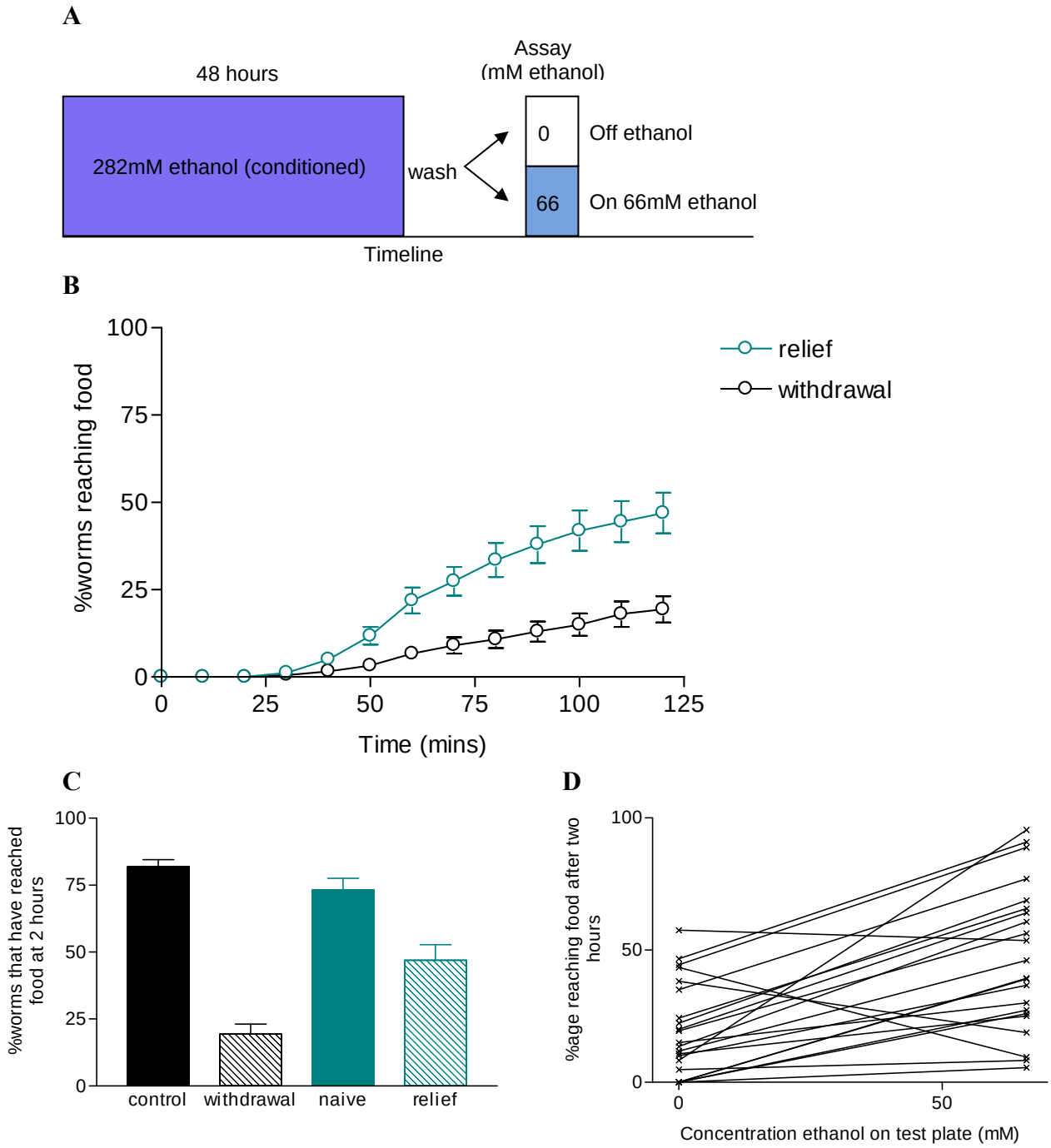


Figure 5.4 Relief from withdrawal: Worms conditioned at 282mM ethanol develop withdrawal when removed from ethanol, which is relieved by 66mM acute ethanol. n=22 (A) Time of the relief from withdrawal experiment. (B) The cumulative percentage of worms reaching the food every ten minutes on either 0mM ethanol (withdrawal) or 66mM ethanol (relief) food race plates. All worms have been exposed to 282mM ethanol for 48 hours before the food race. (C) Bar chart showing the percentage of worms that have reached the food after two hours. Control indicates naive worms tested in the absence of ethanol. Naive indicates naive worms tested at 66mM as a control for the withdrawal relief. (D) Comparison of the 22 independent experiments showing the percentage of worms that have reached the food after two hours on both withdrawal (0mM ethanol) and relief (66mM ethanol) food race plates. A relief from withdrawal effect is present in 19 out of 22 experiments.

When withdrawn animals were tested in the presence of a low (66mM) concentration of ethanol there was an increase in the number of animals reaching the food source within two hours (Figure 5.4). This concentration of ethanol did not significantly affect the performance of ethanol naive animals. This demonstrated the phenomenon of withdrawal relief. A one way ANOVA of the percentage of worms that have reached the food after two hours showed a significant effect ($F_{3,84} = 42.49$, $P<0.0001$). Bonferroni Multiple comparison post-tests showed a significant difference between control and withdrawal ($t=10.22$, $P<0.001$), between withdrawal relief and the same concentration of ethanol applied to naive worms ($t=4.275$, $P<0.001$) and between withdrawal and withdrawal relief ($t=4.511$, $P<0.001$). In 22 independent experiments comparing the performance of withdrawn animals in the food race in the presence or absence of 66mM, only 3 failed to show an improvement on ethanol (Figure 5.4D). This demonstrates that this is an effect of neuroadaptation to ethanol.

5.2.5 Conditioned *C. elegans* show a reduction in body size

It was also considered whether prolonged exposure to ethanol triggered any gross developmental or growth defects that would impair performance of the animals. Indeed it has been reported that chronic exposure to high concentrations of ethanol can impair development (Davis et al., 2008). The comparative size of worms that had experienced 48 hours exposure to 257mM ethanol compared to age-matched control worms was therefore measured.

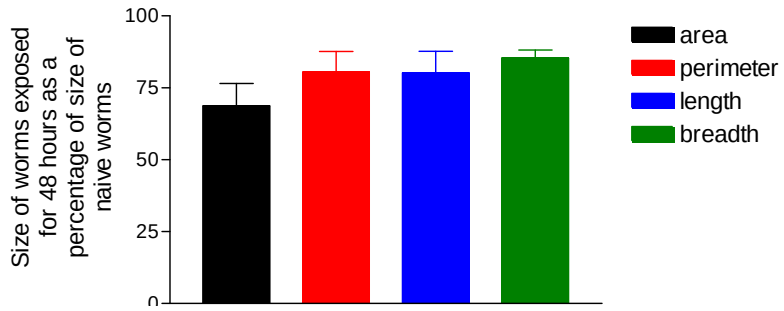


Figure 5.5 Effects of long term exposure to ethanol on worm size as a percentage of normal worm size. n=5.

It was found that the worms exposed to ethanol for 48 hours were significantly smaller in area (t_3=4.015, P=0.0277) and breadth (t_3=5.309, P=0.0130) than the naïve worms (Figure 5.5) as measured by the area in pixels taken up by the worm in photographs of the same magnification. This is consistent with the previous work (Davis et al., 2008), which has demonstrated that chronic exposure to ethanol causes a developmental delay which would be expected to reduce the size of the worms. That study showed that exposure to 200mM or 400mM ethanol throughout life or for 1.5 days beginning at the onset of reproductive maturity reduced worm body size (Davis et al., 2008). Here we show that 2 days exposure to 257mM ethanol reduces body size.

5.2.6 *C. elegans* show a reduction in rate of egg-laying both during and after ethanol conditioning

It was investigated whether behaviours other than the food race were affected by ethanol withdrawal. Rate of egg-laying was severely reduced both during a 48 hour exposure to 257mM ethanol (beginning at L4) and during the 24 hours subsequent to removal from ethanol after this exposure (Figure 5.6). This is consistent with previous work showing both a reduction in the rate of egg-laying during ethanol intoxication

(Davies et al., 2003) and a reduction in total brood size after larval exposure to ethanol (Davis et al., 2008). The latter may indicate that chronic ethanol can cause a permanent developmental defect.

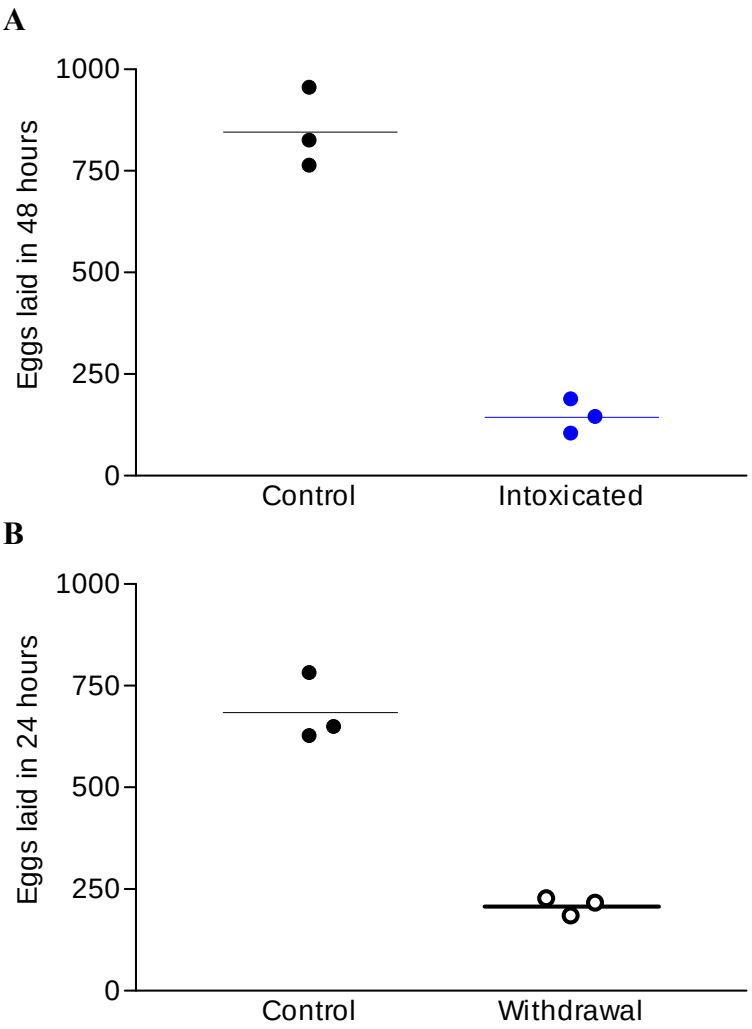


Figure 5.6 Egg-laying is reduced during both intoxication and withdrawal conditions. A. Eggs laid by a developmentally staged population of 10 worms over 48 hour exposure to 257mM ethanol, compared to control. B. Eggs laid in the absence of ethanol over the 24 hour period subsequent to a 48 hour exposure to 257mM ethanol, compared to control.

5.2.7 *C. elegans* show a reduction in rate of body bends during withdrawal but no effect on pumping rate

The question of which behaviours other than the food race were affected by ethanol withdrawal was investigated. A significant decrease in the rate of body bends in the

absence of ethanol after 48 hours exposure to 257mM ethanol was found. This is similar to the effect seen after conditioning with 313mM ethanol in Figure 5.1.

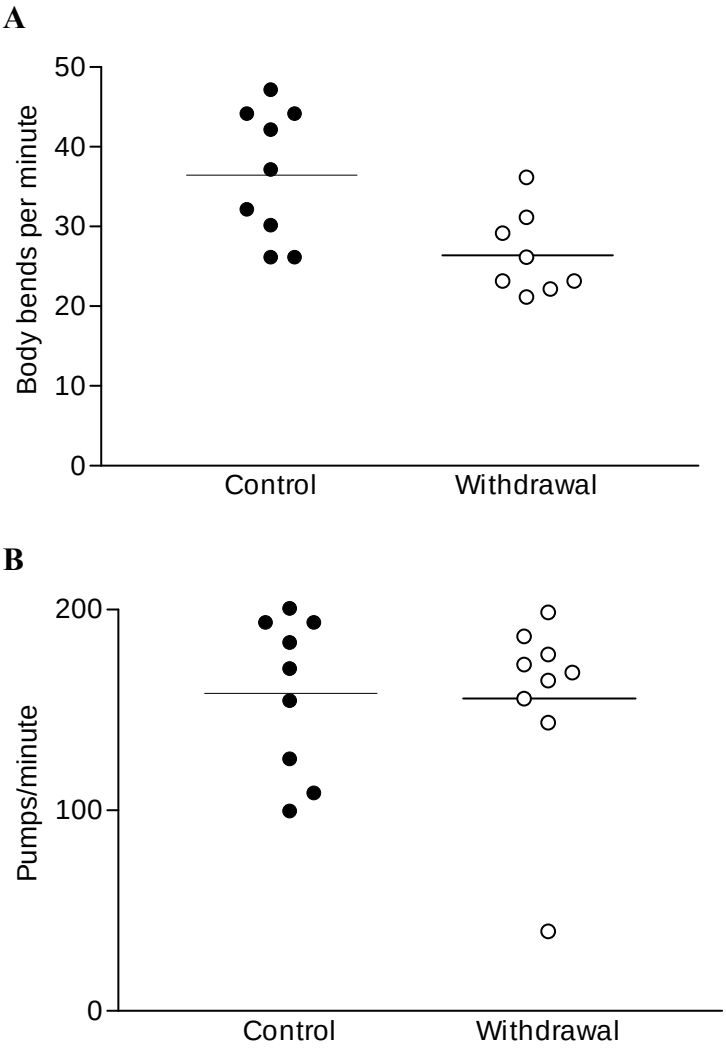


Figure 5.7 Ethanol withdrawal reduces rate of body bends but not pumping rate. A. Body bends per minute in the absence of ethanol after 48 hours exposure to 257mM ethanol, compared to control. B. Pumps per minute in the absence of ethanol after 48 hours exposure to 257mM ethanol, compared to control.

However a significant effect of ethanol withdrawal on rate of pharyngeal pumping behaviour was not seen. Pharyngeal pumping is the rhythmic contraction of the pharynx of the worm in order to draw in and crush the bacteria on which the worm feeds. This is a measure of the feeding rate of the worms.

5.2.8 *C. elegans* recover from the withdrawal effect within 24 hours

As described in section 5.1 there are various reasons for ethanol to cause effects that persist after the removal of ethanol. If the effects that are seen are caused by neuroadaptation to ethanol the worm would be expected to recover over time. This would be due to the affected circuits re-adapting to the absence of ethanol. However if the conditioning procedure had had a toxic effect or caused any kind of permanent developmental defect, the worm would not recover. The response of worms in the food race 24 hours after a 48 hour conditioning period was therefore tested.

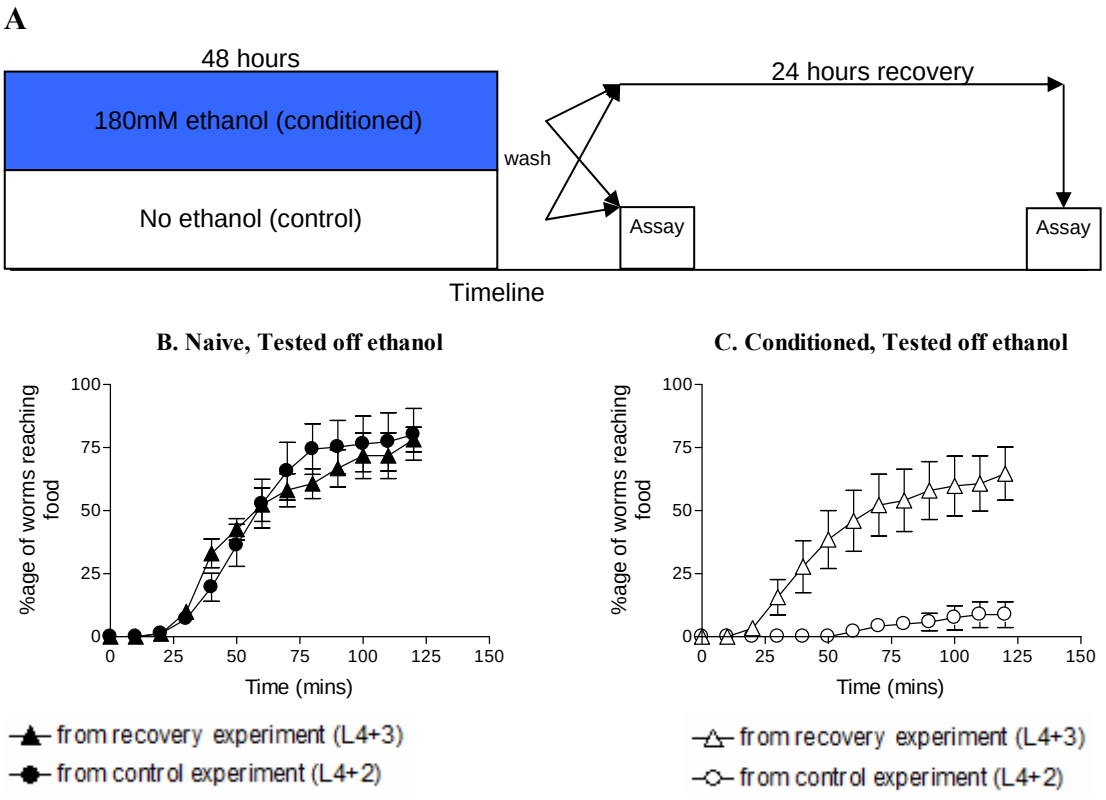


Figure 5.8 Recovery from conditioning. (A) Timeline of the experiment (B) Control worms tested in the food race in the absence of ethanol at L4+2 days and L4+3 days (C) Conditioned worms tested immediately after removal from conditioning plates (L4+2days), or after 24 hours recovery (L4+3days). (D) Bar chart showing the percentage of worms that have reached the food after two hours.

A one-way ANOVA analysing the percentage of worms that had reached the food gave a significant difference ($F_{3,12} = 16.83$, $P<0.0001$). Bonferroni Multiple comparison post-tests showed a significant difference between control and withdrawal immediately after conditioning ($t=6.185$, $P<0.001$) but no difference between control and withdrawal after the 24 hour recovery period ($t=1.175$, $P>0.05$).

This means that immediately after 48 hours ethanol conditioning if worms are removed from ethanol they display ethanol withdrawal as has been previously shown (Figure 5.3). However by 24 hours after removal from ethanol the behaviour of the withdrawn worms is not significantly different to control worms (Figure 5.8). The worms therefore recover completely from the withdrawal effect. The possibility that the conditioning procedure causes a toxic effect or permanent developmental defect which causes part of the withdrawal behaviour can thus be ruled out.

It has thus been clearly demonstrated that *C. elegans* can show intoxication in response to acute ethanol as well as tolerance and withdrawal in response to chronic ethanol. By demonstrating a relief from withdrawal effect it has been shown that the response to chronic ethanol is due to a neuroadaptation to the presence of ethanol in the worm. This adaptation could be causing an alteration in the worm's normal locomotion, an alteration in their ability to navigate towards the food source, an alteration in their ability to detect the food source or most likely a combination of all three. It has also been shown that the conditioning procedure may be causing a slight developmental delay, but it is not causing any irreversible damage to the worm.

5.2.9 Investigating the threshold conditioning concentration required to produce a significant withdrawal relief effect

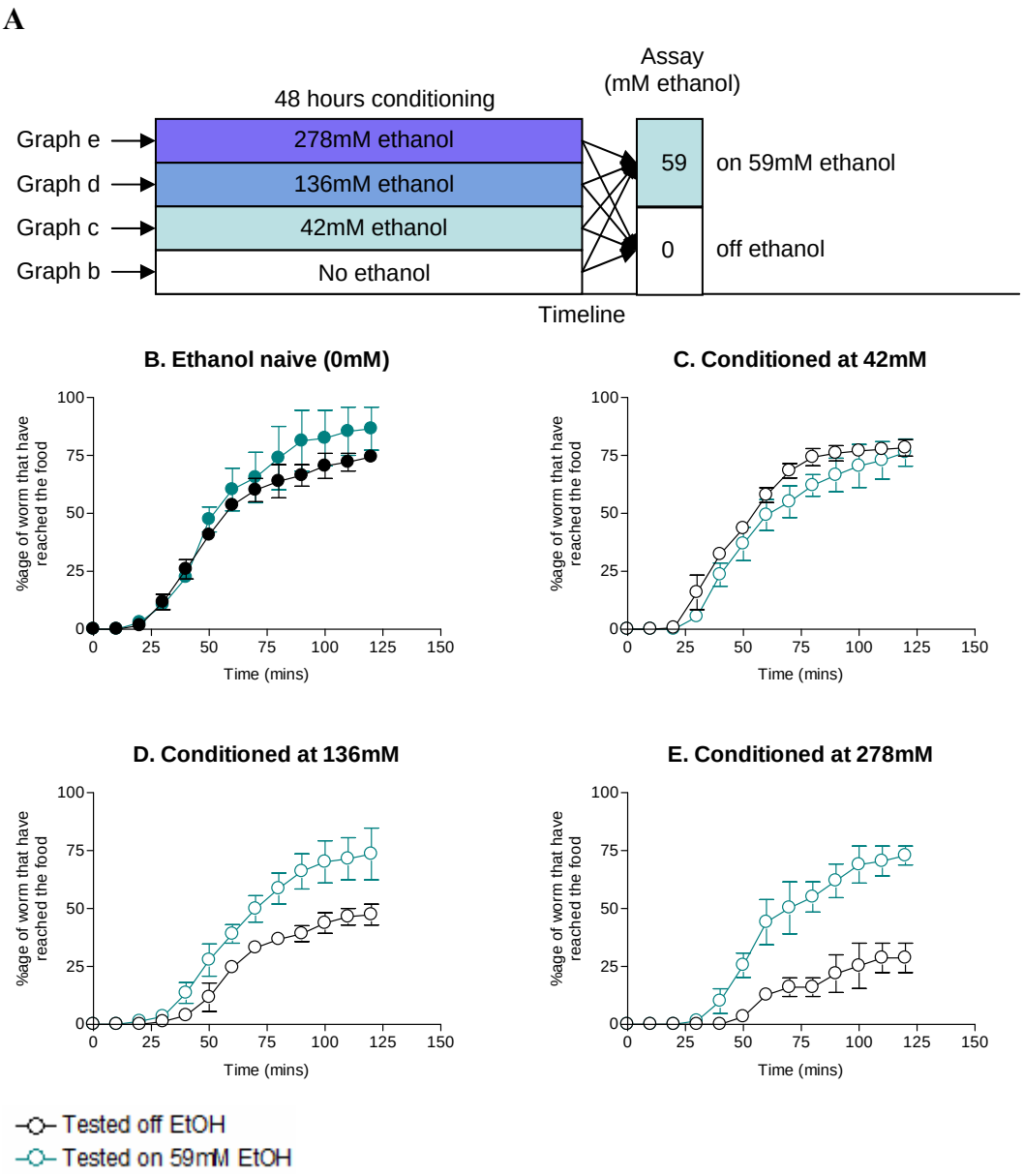


Figure 5.9 Conditioning at various concentrations of ethanol. (A) Timeline of experiment. (B-E) All graphs show tests for withdrawal (tested off ethanol) and relief from withdrawal (tested on 59mM ethanol) after 48hrs conditioning at different concentrations of ethanol.

In order to go on to investigate these effects in more detail, it was first investigated if the effect of any developmental delay on the conditioned worms could be minimised by reducing the conditioning concentration or the length of time the worms are

conditioned for without impacting the ability to detect the tolerance and withdrawal effects.

Figure 5.9 shows that there is no difference between withdrawal (black open circles) and withdrawal relief (green open circles) when conditioned at 42mM (Figure 5.9c). After conditioning at 136mM a difference between withdrawal and withdrawal relief is detectable but this is not significant (Figure 5.9d). Only conditioning at 278mM ethanol (Figure 5.9e) produces a significant difference between withdrawal and withdrawal relief ($t_2 = 5.881$, $P<0.05$).

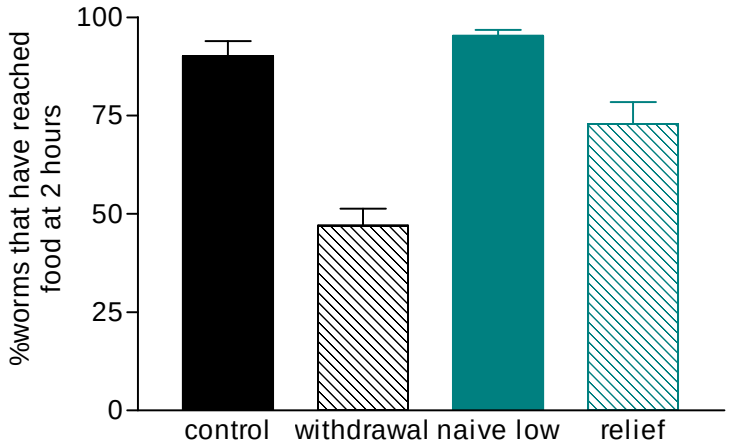
Further investigations will thus continue to use concentrations of ethanol in the range 250mM-350mM to condition worms.

5.2.10 *C. elegans* develop significant withdrawal and withdrawal relief effects after 6 hours conditioning

The effect of reducing the length of time for which the worms were conditioned was investigated. After 6 hours conditioning at 354mM a one way ANOVA of the percentage of worms that have reached the food after two hours showed a significant effect ($F_{3,12} = 28.30$, $P<0.0001$). Bonferroni Multiple comparison post-tests showed a significant difference between control and withdrawal ($t=7.449$, $P<0.001$), between withdrawal relief and the same concentration of ethanol applied to naive worms ($t=3.887$, $P<0.05$) and between withdrawal and withdrawal relief ($t=4.456$, $P<0.01$).

This means that after 6 hours conditioning there is clearly a significant withdrawal effect (Figure 5.10a). However this is not as pronounced as the effect after 48 hours (Figure 5.4 and 5.10b).

A



B

mean percentage worms reaching the food after two hours

	control	withdrawal	naive	relief	% increase relief from withdrawal
after 48 hours conditioning	81.89	19.35	73.11	46.95	142.6357
after 6 hours conditioning	90.13	47.03	95.31	72.82	54.83734

Figure 5.10 The effect of 6 hours ethanol conditioning in the food race. (A) Bar chart showing the percentage of worms that had reached the food after two hours in the food race. Withdrawal and relief worms had been conditioned at 354mM ethanol for 6 hours. Control and Naive low worms were naive to ethanol. Naive low and relief worms were tested on 60mM ethanol food race plates. Control and withdrawal worms were tested in the absence of ethanol. (B) Table showing a comparison between worms conditioned for 48 hours and worms conditioned for 6 hours (from Figure 5.4).

It has been shown above that there is a significant effect of withdrawal and withdrawal relief after 6 hours conditioning, but not after conditioning at lower concentrations. Further investigations will go on to perform a more detailed analysis of the changes in locomotion that give rise to the intoxication, tolerance, withdrawal and withdrawal relief effects seen in the food race assay. For these analyses, the worms will continue to be conditioned at a concentration in the range 250-350mM for a 6 hour period in order to reduce any effect caused by a developmental delay without

losing the neuroadaptation to ethanol. The worms will then be washed and behaviour tested at one of three concentrations of ethanol; high (250-350mM), low (40-90mM) or none (0mM). This will produce six different conditions as previously described.

***Conditioning*\Test**	**No ethanol**	**Low ethanol**	**High ethanol**
No conditioning (naive to ethanol)	Control ●	Naive low ●	Naive high/ Intoxication ●
Conditioning	Withdrawal ▨	Relief ▨	Tolerance ▨

Table 5.1 Explanation of the six conditions under which worm behaviour has been analysed.

5.2.11 Chronic exposure to ethanol initially reduces the rate of reversals in *C. elegans*, irrespective of test ethanol concentration.

As the behavioural readout for the effects of acute ethanol intoxication and for withdrawal were the same i.e. a reduced ability to reach the food source in the food race, it was investigated whether this poor performance results from a similar aberrant locomotory pattern in both acute 'intoxication' and chronic 'withdrawal'. Initial visual inspection indicated that the behavioural effects of ethanol on navigation were complex.

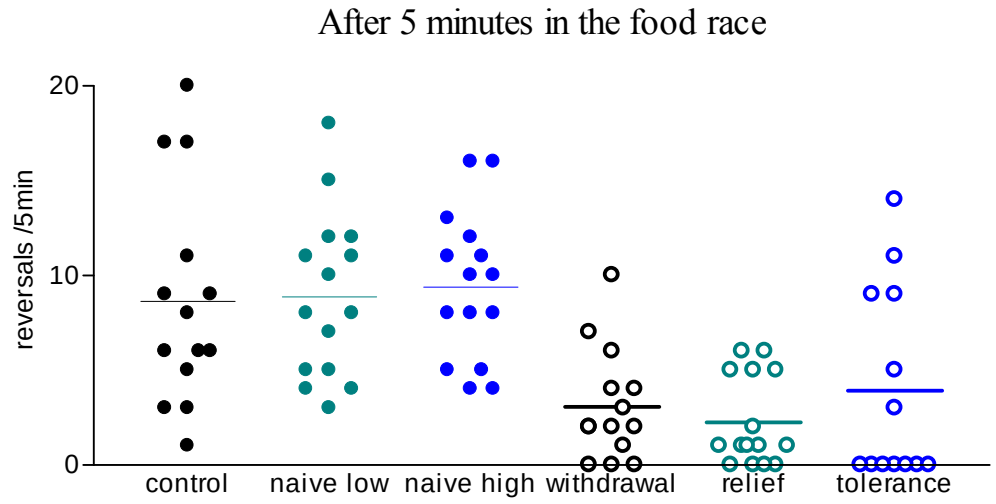
Figure 5.11 Example photographs of *C. elegans*. (A) control (B) intoxicated (C) withdrawn. Scale bar represents 1mm.

Thus, whilst worms acutely exposed to ethanol show a very shallow waveform, uncoordinated body bends and an inability to move forward which correlates with previous descriptions (Davies et al., 2003), animals undergoing withdrawal had a

distinctly different locomotory pattern consisting of deep body bends and numerous turns (Figure 5.11). It was decided to manually quantify these alterations in behaviour.

The first behaviour investigated was the frequency of spontaneous reversals. As described in section 5.1 *C. elegans* navigation in the food race is likely to involve changes in the frequency of spontaneous reversals allowing navigation towards the food. Thus, one of the ways in which ethanol could be affecting the ability of *C. elegans* to reach the food could be by affecting the rate of reversals. In fact, it has been previously shown that the frequency of spontaneous reversals can alter the ability of a worm to navigate towards a food source (Brockie et al., 2001;Zheng et al., 2004).

A



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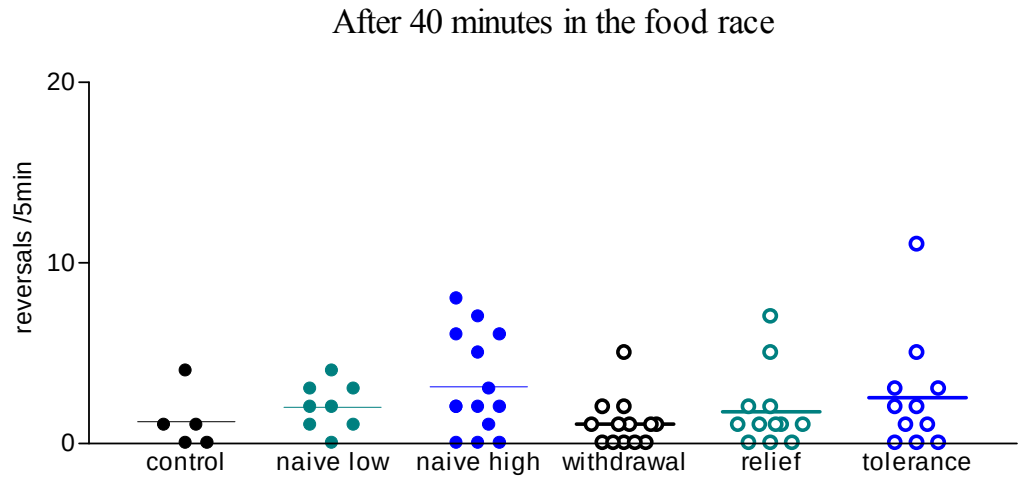


Figure 5.12 Rate of reversals is affected by ethanol conditioning and time on the food race plate. n>=12. The line indicates the mean value. (A) reversals after 5minutes on a food race plate (B) reversals after 40minutes on a food race plate

The number of reversals in 5 minutes was measured, both 5 minutes after adding the worms to the food race plates (see section 2.3.4) and 40 minutes after adding them. These time-points mimic an early point in the food race where none of the worms would be expected to have reached the food and a late point in the food race where more than half of the control worms would have reached the food. In addition if behaviour in the food race is related to the area restricted search behaviour described in section 5.1, then the 5 minute time-point reflects a local search state, whereas the 40 minute time point reflects a dispersal state.

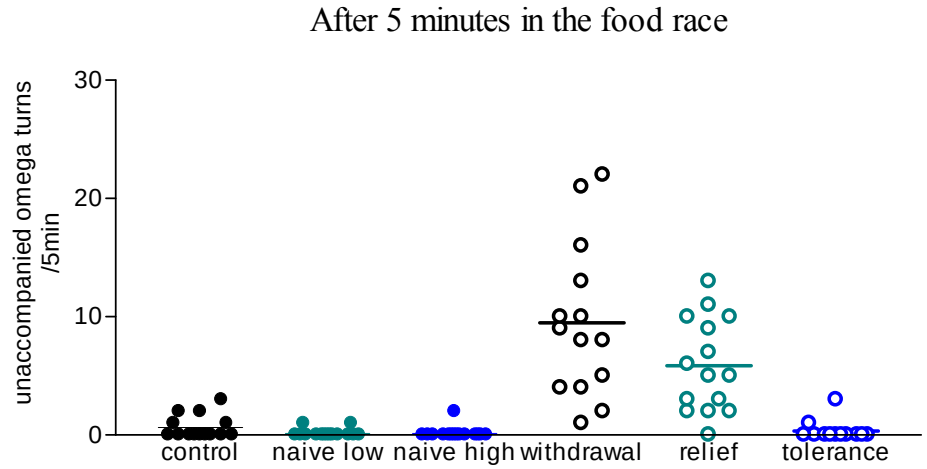
5 minutes after being added to the food plate the frequency of reversals of all the ethanol naive worms, at all doses of acute ethanol, was very similar (approximately 9 reversals in the 5 minute period). The frequency of reversals of all the ethanol conditioned worms was much lower (in the range 2-4 reversals in the five minute period). The effects of ethanol conditioning on rate of reversals did not display the pattern of intoxication improved by tolerance and withdrawal relieved by a low concentration of ethanol described in section 5.1. This therefore does not appear to be an effect caused by neuroadaptation to ethanol.

After 40 minutes on the food race plate the reversal frequency under all conditions was very similar (in the range 1-4 reversals in 5 minutes).

5.2.12 The frequency of unaccompanied omega turns is increased in withdrawn worms and this is relieved by a low concentration of ethanol.

Another behaviour examined was the frequency of unaccompanied omega turns i.e. omega turns that did not occur directly following a reversal. Omega turns were defined as the head nearly touching the tail, or a reorientation of more than 135° in a single head swing (Gray et al., 2005).

(A)



(B)

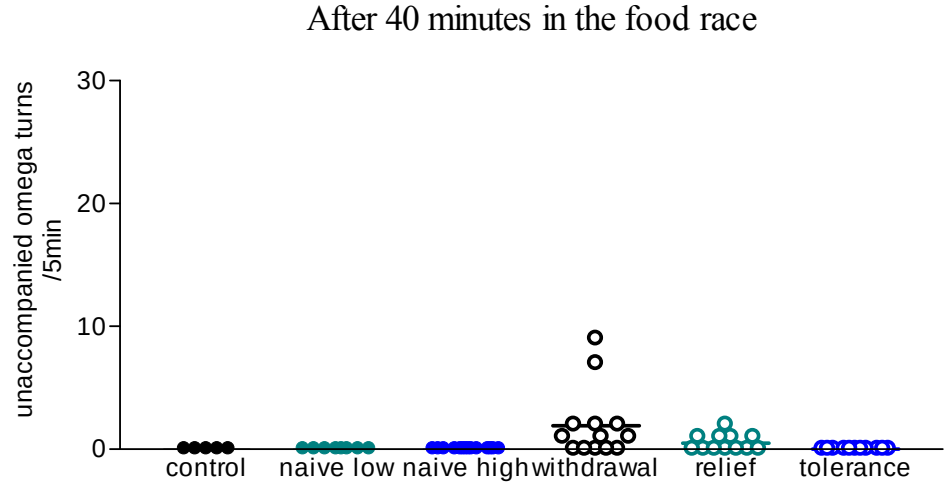


Figure 5.13 Rate of unaccompanied omega turns n>=12 (a) after 5minutes on a food race plate (b) after 40minutes on a food race plate. The line indicates the mean value.

As for reversals, the number of unaccompanied omega turns in 5 minutes was measured, both 5 minutes after adding the worms to the food race plates and 40 minutes after adding them. 5 minutes after being added to the food the rate of unaccompanied omega turns in the control naive low, naive high and tolerance groups was very low (< 2 in the 5 minute period). This is consistent with the previously reported fact that in control worms whilst omega turns do occur alone they are rare and are much more common following a reversal.

However the rate of unaccompanied omega turns in the withdrawn worms was much higher (9.5 in 5 minutes). This was partially relieved by a low concentration of ethanol (withdrawal relief) (5.9 in 5 minutes). A one way ANOVA showed a significant difference ($F_{5,79} = 22.01$, $P<0.0001$). Bonferroni Multiple comparison post-tests showed a significant difference between control and withdrawal ($t=7.343$, $P<0.001$), between withdrawal relief and the same concentration of ethanol applied to naive worms ($t=4.90$, $P<0.001$) and between withdrawal and withdrawal relief ($t=3.064$, $P<0.05$). This indicates that this is a clear effect of neuroadaptation to ethanol.

40 minutes after being added to the food race plates the rate of unaccompanied omega turns had decreased in worms under all the conditions. Worms in the control, naive low, naive high and tolerance groups had no unaccompanied omega turns and worms in the withdrawal and withdrawal relief groups had < 2 in 5 minutes.

5.2.13 Investigating the behaviours following reversals

Spontaneous reversals are usually followed by a change of direction due to increased amplitude of the first forward head swing. Previously published work has established some of the neurons which control the extent of this change in direction (Gray et al., 2005). These changes of direction were classified into 5 categories. These were omega turns defined as above, change direction (a reorientation of 20-135°), slight change direction (a reorientation of <20°), no change or curled into ball (where the worm forms a circle with the head and tail overlapping and remains in that position for at least 0.5 seconds. The percentage of total reversals that were followed by each of these behaviours was then recorded.

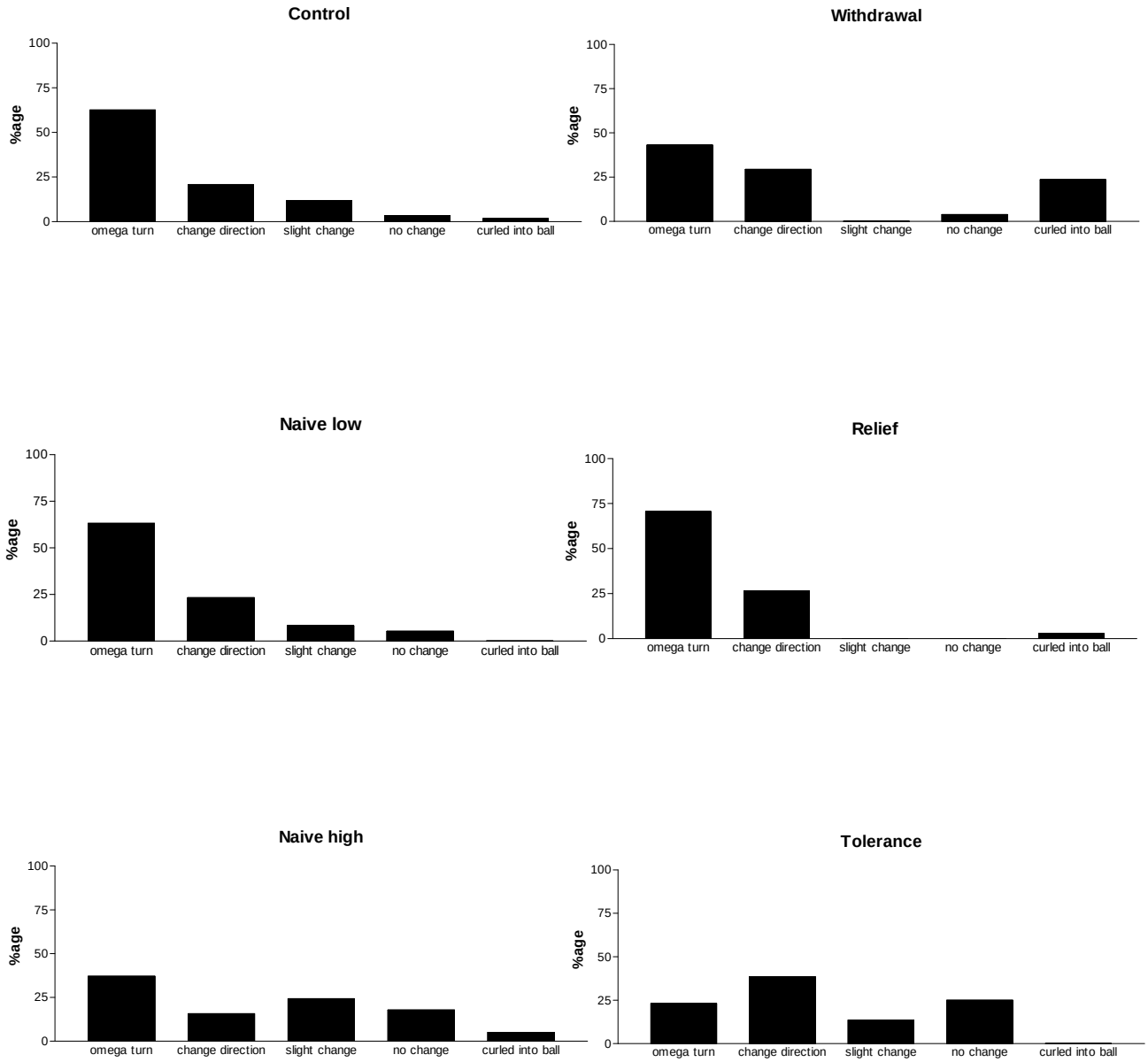


Figure 5.14 Actions following a reversal (percentage of total reversals). The behaviour of worms immediately after a reversal was classified under five descriptions. The likelihood (in %) of a reversal being followed by a given behaviour is shown for each of the six conditions. Data comes from five minute recordings of at least 13 worms per condition, taken five minutes after being placed on a food race plate.

Withdrawn worms have a greater tendency to curl into a ball after a reversal, an effect which is partially relieved by a low concentration of ethanol. Naive high (intoxicated) worms show a reduced likelihood of omega turns following a reversal and an increased likelihood of a slight change of direction or no change at all. Tolerant worms show a similar pattern although they show an even more reduced likelihood of

an omega turn and an increased likelihood of a medium change in direction compared to control as well as an even more increased likelihood of no change than in intoxicated worms. This does not necessarily indicate the presence of a tolerance effect, which one would expect to return the distribution towards the control distribution.

5.2.14 Ethanol conditioning does not affect reversal length

The length of backward movement during the spontaneous reversals was measured. This length was measured by the number of head turns the worm made during the backwards movement. A head turn was defined as a change in the direction of curvature or the area immediately behind the pharynx. Neither acute ethanol nor ethanol conditioning affected the percentage of reversals that are three of more head turns in length.

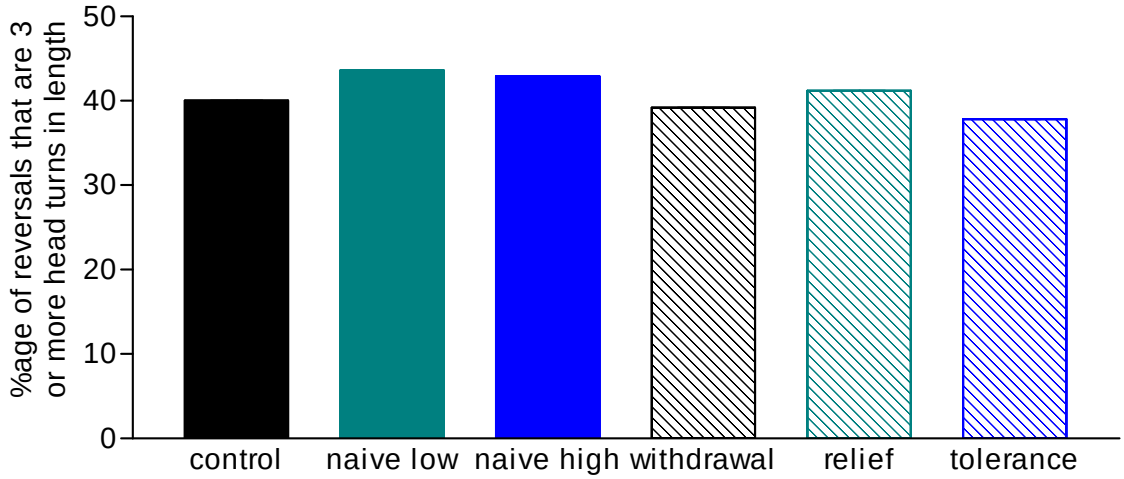


Figure 5.15 Ethanol conditioning does not affect length of reversals. Data comes from five minute recordings of at least 13 worms per condition, taken five minutes after being placed on a food race plate.

5.2.15 Ethanol withdrawal alters the association of omega turns with longer reversals

It has been previously observed that omega turns are more commonly coupled to reversals of three of more head swings (Gray et al., 2005). The percentage of all reversals of three of more head swings that are followed by an omega turn was measured and compared to the percentage of shorter reversals that are followed by an omega turn.

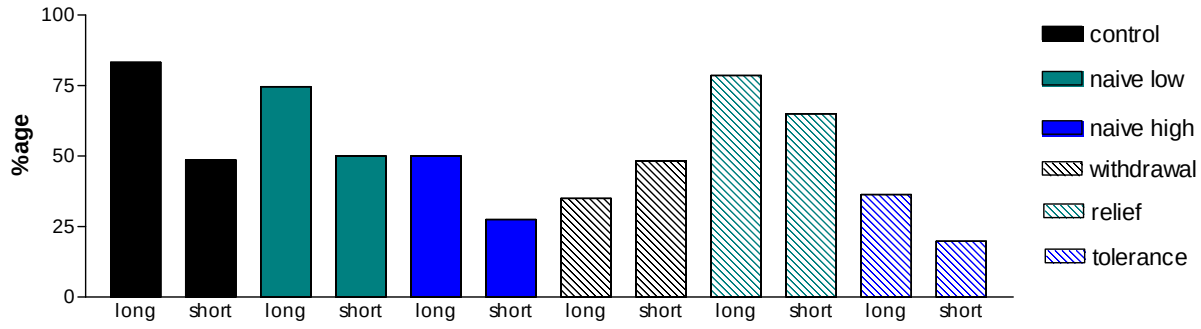


Figure 5.16 The likelihood of a long reversal being followed by an omega turn compared to the likelihood of a short reversal being followed by an omega turn under each of the six conditions. Data comes from five minute recordings of at least 13 worms per condition, taken five minutes after being placed on a food race plate.

It can be seen that the control worms agree with the previously reported observation. In the control worms a long reversal has an 83% chance of being followed by an omega turn, whereas a short reversal only has a 49% change of being followed by an omega turn. However in the withdrawn worms long reversals are less likely to be followed by an omega turn than are short reversals. In the relief from withdrawal worms short reversals are less likely to be followed by an omega turn than long reversals but the difference is less marked than in the control indicating that this may be an effect of neuroadaptation to ethanol.

This indicates that in the circuit which controls the likelihood of an omega turn occurring following a reversal, there is an effect of acute ethanol as intoxicated worms show less omega turns following a reversal (Figure 5.14). In addition there is an interaction between the effect of reversal length on this circuit and ethanol withdrawal as ethanol withdrawal reverses the association of omega turns with longer reversals (Figure 5.16).

5.2.16 Ethanol withdrawal increases the loopyness of worm locomotion

Automated analysis software designed by Christopher James (ISVR, University of Southampton) was used to analyse video capture images of *C. elegans* and thus extract data from approximately 20 worms filmed under each of the six conditions. These videos were taken 5 minutes after the worms were placed onto a food race plate. This video analysis system extracts the xy coordinates of ten node centres along the length of the worm in every frame of the video by a method described in section 2.10.2. These can act as a model for the behaviour of the worm, and can be used to extract several characteristics of the worm's movement. The loopyness, efficiency and speed of the worm's motion has been analysed (see sections 2.9.1.4-2.9.1.7 for definitions) and represented the range of morphology of body topology using a cluster analysis (see section 2.10.2).

The loopyness of the worm's shape in a frame can be calculated as the mean perpendicular distance of the ten node centres from a linear regression line drawn between them (see section 2.10.2). The mean loopyness of each worm in each video can then be plotted.

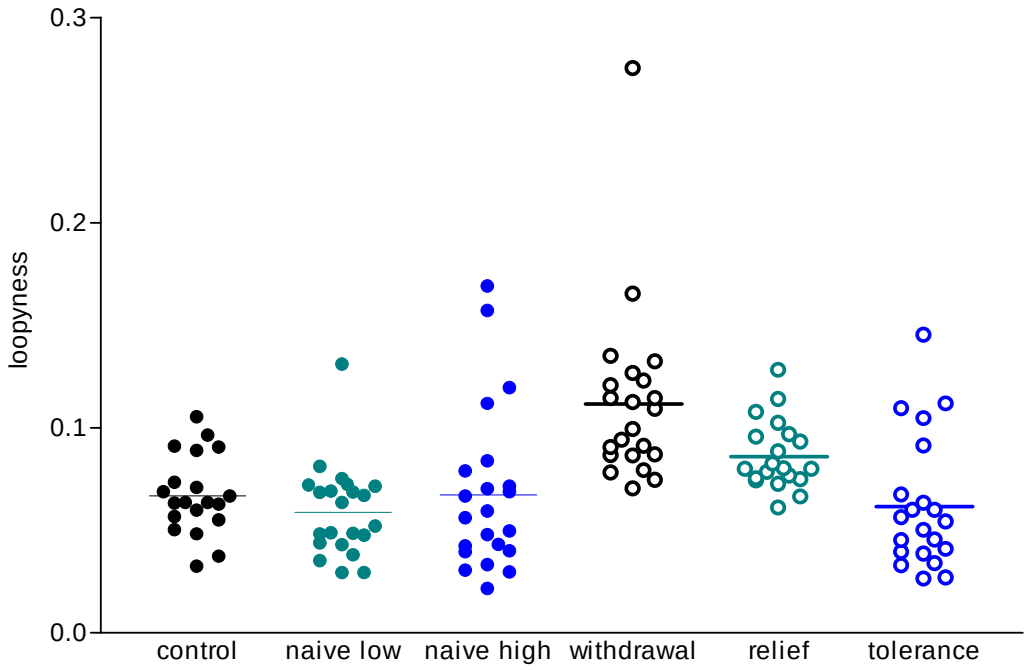


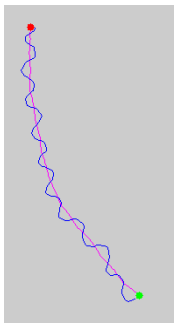
Figure 5.17 Mean loopyness of each worm over 30 second video filmed five minutes after worm was added to food race plate. See section 2.10.2 for definition and method of calculation of loopyness. n>=20. The line indicates the mean value.

Ethanol withdrawal significantly increases the loopyness of the worm's locomotion. This is partially relieved by a low dose of ethanol. A one way ANOVA showed an overall significant difference ($F_{5,121} = 9.173$, $P<0.0001$). Bonferroni Multiple comparison post-tests showed a significant difference between control and withdrawal ($t=4.683$, $P<0.001$), but not between withdrawal relief and the same concentration of ethanol applied to naive worms ($t=2.837$, $P>0.05$) or between withdrawal and withdrawal relief ($t=2.678$, $P>0.05$). This agrees with the data on unaccompanied omega turns. This measure therefore provides a correlate of withdrawal and relief, but not intoxication or tolerance.

5.2.17 Both ethanol withdrawal and intoxication decrease the efficiency of worm locomotion

The efficiency of worm locomotion can be described by dividing the distance travelled by the centre of mass of the worm by the distance of the sinusoidal path that the worm actually covers (see section 2.10.2).

A



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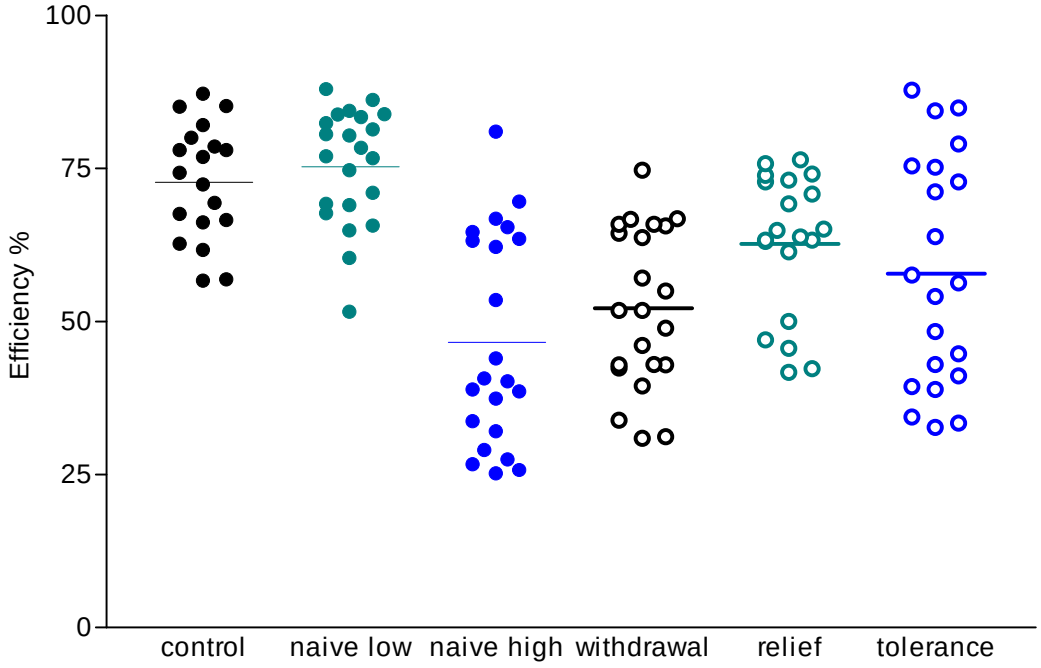


Figure 5.18 Efficiency of worm locomotion (A) Track showing the distance the worm actually covers (in blue) compared to the distance travelled by its centre of mass (in pink). Efficiency is calculated as the pink line divided by the blue line (shown here as a percentage). See section 2.10.2 for definition and full method of calculation of efficiency (B) Efficiency of worm locomotion in a 30 second video taken five minutes after the worm was added to the food race plate. n<=20. The line indicates the mean value.

Both intoxication and withdrawal decrease the efficiency of the movement of the worm. The decrease in efficiency in withdrawal is partially relieved by a low concentration of ethanol. A one way ANOVA showed an overall significant difference ($F_{5,121} = 14.69$, $P<0.0001$).

Bonferroni Multiple comparison post-tests showed a significant difference between control and intoxication (naive high) ($t=6.206$, $P<0.001$) and also between tolerance and control ($t=3.529$, $P<0.01$), but not between intoxication and tolerance ($t=2.671$, $P>0.05$)

There was also a significant difference between control and withdrawal ($t=4.909$, $P<0.001$), but not between withdrawal relief and the same concentration of ethanol applied to naive worms ($t=2.962$, $P>0.05$) or between withdrawal and withdrawal relief ($t=2.480$, $P>0.05$).

This measure therefore provides a correlate of intoxication, withdrawal and withdrawal relief but not tolerance.

5.2.18 Both ethanol intoxication and withdrawal decrease the speed of worm locomotion

The speed of the worm on plates is calculated by the distance travelled by its centre of mass over time (see section 2.10.2). Both ethanol intoxication (naive high) and withdrawal decrease the speed of worm locomotion although it is decreased to a greater extent in intoxication. A low concentration of ethanol partially relieves this effect in the withdrawn worms. However, tolerance is not significantly different to

intoxication. A one way ANOVA showed an overall significant difference ($F_{5,121}$ = 33.05, P<0.0001).

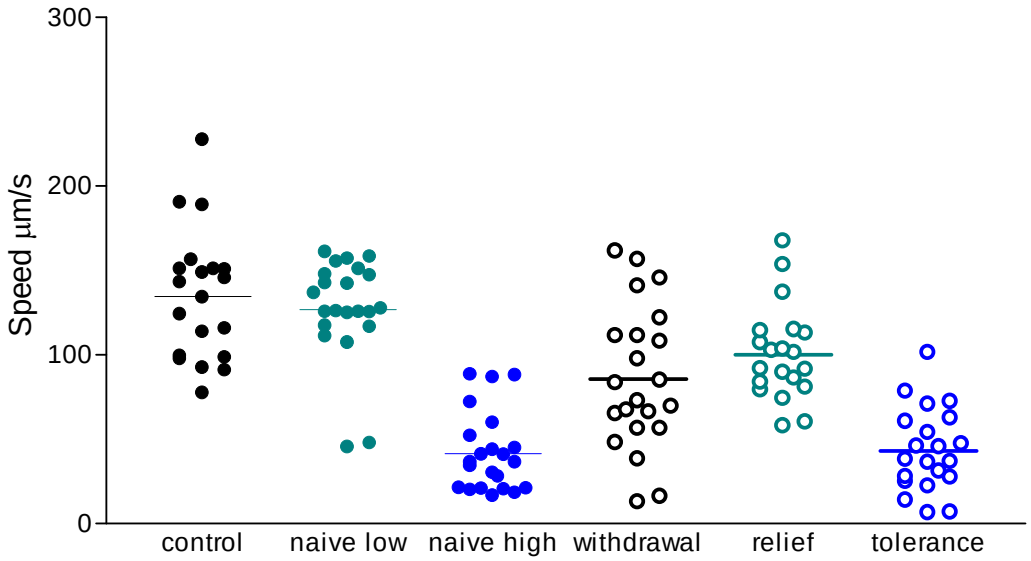


Figure 5.19 Speed of worm locomotion in a 30 second video taken five minutes after the worm was added to the food race plate. n<=20. See section 2.10.2 for full method of calculation of speed. The line indicates the mean value.

Bonferroni Multiple comparison post-tests showed a significant difference between control and intoxication (naive high) (t=9.401, P<0.001) and also between tolerance and control (t=9.134, P<0.001) but not between intoxication and tolerance (t=0.1665, P>0.05)

There was also a significant difference between control and withdrawal (t=4.932, P<0.001), but not between withdrawal relief and the same concentration of ethanol applied to naive worms (t=2.695, P>0.05) or between withdrawal and withdrawal relief (t=1.463, P>0.05). This measure therefore provides a correlate of intoxication, withdrawal and withdrawal relief, but not tolerance.

Ethanol withdrawal causes a reduction in both efficiency and speed which is relieved by a low concentration of ethanol. Ethanol intoxication causes a reduction in both efficiency and speed which is not improved in worms previously exposed to ethanol.

These two measures are interrelated as speed is the distance travelled by the centre of mass over time, whilst efficiency is the distance travelled by the centre of mass over the distance of the worm tracks. They are thus both measures of the worms' ability to cover distance in a normal manner.

5.2.19 Cluster analysis

The cluster analysis produces a spread of data points such that points close together represent similar worm shapes and points far apart from each other represent very different worm shapes (see section 2.10.2). By plotting worms under different conditions onto the cluster analysis it can be seen whether these conditions affect the distribution of body shapes that a worm can display.

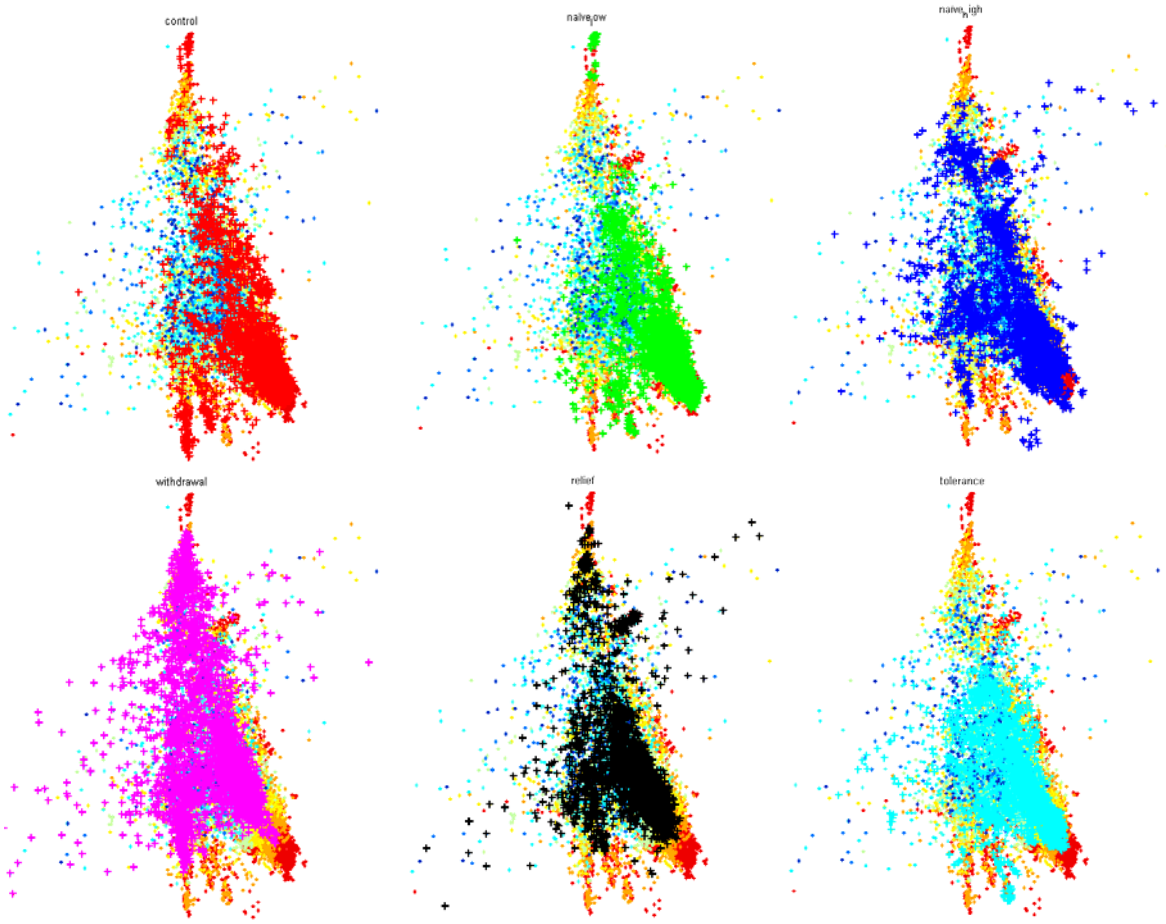


Figure 5.20 Cluster analyses. Points close together represent similar worm shapes and points far apart from each other represent very different worm shapes. Worms from each condition are plotted onto the same layout of all the worms together. Control = red, Naive low = green, Naive high = blue, Withdrawal = pink, Relief = black and Tolerance = light blue.

Whilst this cluster analysis is still under development it could in principle be used to show how both intoxication and withdrawal affect the range of body shapes that a worm displays. At the moment it can be seen that intoxication and withdrawal do alter the range of body shapes. It is not obvious whether worms previously exposed to ethanol (tolerance) are less affected than acutely intoxicated (naive high) worms, or whether a low dose of ethanol (relief) relieves the withdrawal effect. This analysis does not at present tell us which body shapes are affected by the changes, however in the future this analysis may be refined in order to discover that. This provides further evidence that ethanol affects the locomotion of *C. elegans*.

5.2.20 Summary

Behaviour	Effect of Intoxication	Improved by tolerance?	Effect of withdrawal	Relieved by ethanol?
Food race (%worms reached food in 2 hours)	↓	y	↓	y
Frequency of egg laying	↓	-	↓	-
Frequency of reversals	no change	-	↓	n
Frequency of unaccompanied omega turns	no change	-	↑	y
Likelihood of omega turn following a reversal	↓	n	↓	y
Likelihood of slight or no change of direction following reversal	↑	n	↓	n
Likelihood of curling into a ball after a reversal	↑	y	↑	y
Length of reversals	no change	-	no change	
Ratio of likelihood of an omega turn following a long reversal to likelihood of an omega turn following a short reversal	no change	-	↓	y
Loopyness	no change	-	↑	y
Efficiency	↓	n	↓	y
Speed	↓	n	↓	y
Change in body morphologies seen in cluster analysis	y	-	y	-

Table 5.2 Summary of results presented in Chapter 5

5.3 Discussion

5.3.1 *C. elegans* show neuroadaptation in response to chronic exposure to ethanol

Results described in this chapter have demonstrated that *C. elegans* show neuroadaptation in response to chronic exposure to ethanol. It has previously been established that *C. elegans* undergoes intoxication in response to acute ethanol (see Chapter 3 for summary). Worms have been conditioned for 48 hours with approximately 300mM ethanol, which is a concentration at which the inhibition of locomotion by ethanol is approximately half-maximal (see Chapter 3). It has then been demonstrated that, if a conditioned worm and a naive worm are observed when exposed to the same intoxicating concentration of ethanol, the conditioned worms show significantly less of an inhibition in the food race assay (Figure 5.2). This indicates that they have become tolerant to the effects of ethanol.

It has also been shown that, if a conditioned worm and a naive worm are observed in the absence of ethanol, the conditioned worm behaves significant differently to the naive worm in the food race assay (Figure 5.3). This indicates that worms adapt to chronic ethanol producing a distinct behavioural state that exhibits features of withdrawal. Further it has been established that a low concentration of ethanol can partially return this behavioural state to that of control worms (Figure 5.4). This is defined as a relief from withdrawal effect and strongly supports the contention that the conditioned worms are undergoing neuroadaptation to ethanol as discussed in section 5.1.

An alternative explanation for the withdrawal effect could be that the worms were being affected by residual ethanol, despite the fact that all the worms were washed for at least two minutes (see section 2.11.1 - Determining the time required to remove residual ethanol from the worm). However, this is not consistent with the relief from withdrawal effect where the conditioned worms are able to move better when exposed to a low concentration of ethanol than when removed entirely from ethanol.

An alternative explanation for the effect of intoxication in the food race might have been that the ethanol provided a food source for the worms that made them less likely to move towards the point source of *E. coli* OP50. However the observations of tolerance and relief from withdrawal in the food race assay argue against this explanation. Tolerant worms are more likely to move towards the food than intoxicated worms despite having the same concentration of ethanol in the agar. Relief from withdrawal causes worms to be more likely to move towards the food than withdrawn worms despite having a greater concentration of ethanol in the agar.

Tolerance in mammalian systems can be separated into tolerance caused by increased liver clearance of ethanol (dipositional tolerance) which can double in dependant patients and tolerance due to adaptation in the CNS (functional tolerance), which plays a much greater part of the total tolerance effect. Functional tolerance can be separated into acute 'within session' tolerance or rapid and chronic 'between session' tolerance. Acute tolerance was originally defined as occurring when the same concentration of ethanol causes a much greater intoxication when blood ethanol concentration is ascending than when it is descending. Rapid tolerance is seen on the second exposure to ethanol after a single, acute, high concentration exposure. Chronic

tolerance, which is seen in alcoholism, is an effect of repeated or long term exposure to ethanol (Koob and Le Moal, 2006). In *Drosophila* both rapid and chronic forms of functional tolerance have been described, which require octopamine signalling and protein synthesis respectively (Berger et al., 2004) (see Introduction section 1.10.3).

In *C. elegans* it has been previously demonstrated that wild type worms show a modest but significant acute tolerance effect as assayed by speed on agar plates within a 50 minute period (Davies et al., 2004a). In this study this is not observed in the thrashing assay over a three hour period (see Chapter 6, Figure 6.12); however this could be due to differences between the assays. In this context it has been recently shown that thrashing and crawling on plates are distinct forms of locomotion distinguished by distinct kinematics and different underlying patterns of neuromuscular activity (Pierce-Shimomura et al., 2008). Results shown here have demonstrated the presence of a chronic tolerance effect in *C. elegans* seen after a long term ethanol exposure (Figure 5.2).

Only one previous study has looked at the effect of longer term exposure to ethanol on *C. elegans*. In this *C. elegans* were exposed to 350mM ethanol for 18-22 hrs and then removed from ethanol, which are conditions which this study has confirmed would produce a withdrawal effect (Figures 5.3 and 5.8). The worms were then placed on a food plate and their behaviour was observed. It was seen that N2 *C. elegans* displayed social feeding behaviours such as aggregating on the edges of the bacterial lawn, which under normal circumstances they do not. This was shown to be an effect of the NPR-1 pathway (Davies et al., 2004a).

This study did not investigate whether this behaviour was relieved by acute ethanol so it is unclear whether this is a neuroadaptation to the presence of ethanol or an effect of an environmental or cellular stress pathway, inhibition of feeding or a developmental delay as discussed in the Introduction (see section 1.11.5). However the association of NPR-1 with the development of tolerance (Davies et al., 2004a) makes it likely that this is a withdrawal effect (see sections 6.2.4 and 6.3.3.2 for a full discussion of this paper and the effects of the NPR-1 neuropeptide receptor).

As the assays described above are conducted on food race plates rather than food plates, these feeding behaviours would not be expected to affect them; however it would be interesting to see how an *npr-1* mutant behaves in the food race. This is investigated in the next chapter (see sections 6.2.4 and 6.3.3.2).

In conclusion this chapter demonstrates for the first time a chronic tolerance effect and a withdrawal effect confirmed by the presence of relief from withdrawal in the same assay. Neuroadaptation in response to chronic ethanol exposure has thus been shown in *C. elegans*. The fact that *C. elegans* undergo neuroadaptation to the presence of ethanol resulting in tolerance and withdrawal means that they can be used as a model for these aspects of alcohol dependence.

5.3.2 *C. elegans* experience a developmental delay in response to chronic exposure to ethanol but this does not explain the withdrawal effect

Conditioned worms are significantly smaller than naive worms (Figure 5.5). This could be the result of a developmental effect. This is not a permanent effect as *C.*

elegans recover from withdrawal within 24 hours (Figure 5.8). Nevertheless it could still be a developmental delay. This would be consistent with previously published results (Davis et al., 2008), which have reported that chronic exposure to ethanol during larval development temporarily delayed growth, and even chronic exposure to ethanol beginning in adulthood reduced worm body length after 1.5 days exposure. It could therefore be possible that the withdrawal behaviour was the result of this developmental delay, as this would produce an effect of ethanol conditioning that persisted after removal of ethanol.

Other factors that could be involved are oxidative stress, or the activation of cellular stress pathways. In mammalian systems ethanol has been shown to cause a dose-dependent increase in the production of reactive oxygen species and a dose dependent increase in heat shock protein levels (Russo et al., 2001). In *C. elegans* as well exposure to ethanol has been shown to cause upregulation in heat shock protein genes (Kwon et al., 2004). These factors could produce an effect of ethanol conditioning that persisted after removal of ethanol

However, none of these possibilities would explain the withdrawal relief effect in which identically treated worms can perform better in the presence of a low concentration of ethanol. This relief from withdrawal makes neuroadaptation to the presence of ethanol the most likely explanation for withdrawal behaviour. Nonetheless it is possible that, as a low dose of ethanol does not completely relieve the reduction in the ability of the worm to reach the food, this could be due to a combination of the withdrawal effect, the developmental delay and possibly also an effect of oxidative stress or the activation of cellular stress pathways. It has been

shown that in *Drosophila* a cellular stress pathway is, in fact, involved in the development of tolerance to ethanol (Scholz et al., 2005).

5.3.3 Conditioning occurs at concentrations of ethanol that severely inhibit *C. elegans* locomotion

The threshold conditioning concentration required to produce a significant difference between withdrawal and withdrawal relief after 48 hours conditioning is between 136 and 278mM ethanol. 136mM ethanol appears to produce a slight non-significant effect of withdrawal and withdrawal relief. It is possible that a longer exposure to this concentration of ethanol would produce a significant difference between withdrawal and withdrawal relief.

In Chapter 4 evidence was provided that the *C. elegans* cuticle does not seem to be a significant diffusion barrier for ethanol when measuring the behavioural consequences of ethanol exposure. It was suggested that concentrations causing even a slight reduction in locomotion should be considered to be equivalent to sedation in humans and concentrations equivalent to intoxication are likely to be in the <100mM range.

Thus, the concentrations required to develop a measurable neuroadaptation to ethanol in the assays described here would be considered to be equivalent to sedation in humans. Whilst the development of alcohol dependence in the human would be expected to require heavy drinking, these concentrations are probably higher than one would ideally use in the worm to model this alcohol dependence.

The development of alcohol dependence in humans involves repeated withdrawal from ethanol (Duka et al., 2004) and can sometimes develop over decades. It is possible that lower concentrations of ethanol would cause a conditioning effect in *C. elegans* if applied repeatedly and/or for a longer period of time. It is also possible that they are causing a conditioning effect but one that is too slight to be detected by these assays. As with intoxication, in humans, only very severe alcohol withdrawal causes major incapacitation. Milder alcohol withdrawal causes sweating, tremor, sleep disturbance and craving for alcohol (Saitz, 1998). One would expect this to cause much more subtle effects in the worm than the inability to find food in the food race assay.

However the clear development of tolerance and withdrawal in the assays described provides a useful basis for investigating the mechanisms by which neuroadaptation to the presence of ethanol occurs.

5.34 Reversal behaviour in control worms is similar to previously published data

As was described in section 5.1 two behaviours have been described that may be relevant to the locomotion of *C. elegans* after being placed in the food race. These are the biased random walk seen in *C. elegans* chemotaxis (Pierce-Shimomura et al., 1999) and area restricted search seen when *C. elegans* are removed from food and placed in an environment where food is distant. These behaviours are interrelated as they are both part of *C. elegans* strategy for finding food (Gray et al., 2005).

In the assays described in this chapter worms have been removed from food directly placed onto food race plates in which an attractant (food) is present, but far away from the worms so both processes may be relevant to their behaviour. The frequency of reversals was measured at 5 minutes and 40 minutes. These time-points mimic an early point in the food race where none of the worms would be expected to have reached the food and a late point in the food race where more than half of the control worms would have reached the food. In addition if behaviour in the food race is related to the area restricted search behaviour described in section 5.1, then the 5 minute time-point reflects a local search state, whereas the 40 minute time point reflects a dispersal state.

After 5 minutes the control animals' reversal frequency was approximately 9 reversals per 5 minute period, but after 40 minutes it had decreased to approximately 1 reversal per 5 minute period (Figure 5.12). This would be consistent with both behaviours described above. Gray et al. reported approximately 1 short reversal and 1.5 long reversals per minute from 6 to 11 minutes after removal from food (from graph). This would produce approximately 12.5 reversals in 5 minutes which is slightly higher than our measurement of 9 reversals per 5 minutes. From 36-41 the frequency of short or long reversals reported by Gray et al. had decreased to less than ¼ of a reversal per minute (from graph). This would be consistent with the results shown here (Gray et al., 2005). The frequency of reversals would be expected to decrease over time in the biased random walk as the worms orientated themselves towards the chemoattractant. It would also be expected to decrease if the worms had moved from local search behaviour (5 minutes after removal from food) to dispersal behaviour (40 minutes after removal from food).

It has been shown that reversals are commonly followed by a change in direction which may be an omega turn. In control animals omega turns were most commonly coupled to reversals of three or more head swings. Omega turns could occur in isolation but this was rare, they were more commonly coupled to reversals (Gray et al., 2005). This is replicated by the results shown here for control worms after 5 minutes in the food race. A total of 95% of reversals are followed by some type of change in direction (Figure 5.14). 63% of reversals are followed by an omega turn which correlates with the reported high frequency of omega turns in local search behaviour, but that there is less than one unaccompanied omega turn per 5 minute period (Figure 5.13). 40% of all reversals are long reversals, which correlates with the described high frequency of both long and short reversals (Figure 5.15). In addition 83% of long reversals were followed by an omega turn compared to only 49% of short reversals which correlates with the statement that omega turns were more commonly coupled to reversals of three or more head swings (Figure 5.16). The frequency of total omega turns described by Gray et al. is approximately 1.5 per minute (Gray et al., 2005). This would be 7.5 in five minutes, which is again slight higher than our measurement of 6.1±1.2 total omega turns per five minute (data not shown).

Thus the reversal behaviour of the control worms in these assays was similar to the previously described behaviour of *C. elegans* except that the rate of reversals and omega turns was slightly lower than previously described. This could indicate that this is not a pure area restricted search effect.

5.3.5 Reversal frequency is affected by the conditioning procedure, but this is not an effect of neuroadaptation to ethanol

Reversal frequency is affected by the ethanol conditioning procedure (Figure 5.12). However, were this to be an effect of neuroadaptation one would expect to see intoxication having an effect which was improved by tolerance and withdrawal having an opposing effect which was relieved by a low concentration of ethanol. What is seen in Figure 5.12 is that all the conditioned worms, whether tested subsequently with or without ethanol, have a similar low frequency of reversals and all the unconditioned worms have a high frequency of reversals which is similar to each other and not to the conditioned worms. Therefore, this is unlikely to be an effect of neuroadaptation to the presence of ethanol. However it is clearly an effect of the conditioning procedure and an effect which is likely to affect the ability of the worms to navigate towards the food. This alteration in reversal frequency may be the reason that relief from withdrawal does not fully rescue the withdrawal effect. Although one piece of evidence that makes this unlikely is the fact that *glr-1* mutants which have a reduced rate of reversals have been shown to perform normally in the food race (Zheng et al., 2004).

This is unlikely to be a direct effect of a developmental delay as it has been shown that reversal frequency tends to decrease with age between L4 and 3 day adults (Zhao et al., 2003). It could be that this alteration in reversal frequency is caused by an activation of stress pathways in response to prolonged exposure to ethanol. It has been shown that exposure to ethanol causes activation of heat shock family genes in *C. elegans* (Kwon et al., 2004). Perhaps if activation of heat shock pathways did cause a reduction in the rate of reversals, this would cause worms to leave areas in which

something toxic was present that was activating these pathways, which would make evolutionary sense.

There are several genes and neurons that have been shown to affect to frequency of reversals. Dopamine and glutamate have been shown to be involved in area-restricted search in *C. elegans.* Loss of function mutations in *eat-4* which encodes that *C. elegans* ortholog of the mammalian glutamate transporter, *glr-1* which encodes a non-NMDA ionotropic glutamate receptor subunit, and *cat-2* which encodes tyrosine hydroxylase an enzyme required for dopamine synthesis, all produce reduced frequencies of high angled turns at five minutes after being removed from food (Hills et al., 2004). Loss of function of *nmr-1* which encodes an NMDA-type ionotropic glutamate receptor also reduces reversal frequency by a different pathway to loss of function of *glr-1* (Brockie et al., 2001). It is therefore a possibility that the reduction in reversal frequency of conditioned worms is mediated through a dopaminergic or glutamatergic pathway.

A circuit for navigation in *C. elegans* has been described (Gray et al., 2005) which controls the switch between local search and dispersal behaviour. This consisted roughly of three layers of interneurons. The majority of output from the amphid sensory neurons was directed onto layer 1 interneurons (AIA, AIB, AIY and AIZ) which appear to control large scale exploratory behaviours such as movement on food, local search or dispersal. These in turn mostly directed their output onto level 2 (RIA and RIB interneurons and RIM and SMB head motor neurons) which largely directed their output onto level 3 (head interneurons and motor neurons SAA, RIV, RMD, SMD, SIA, SIB and the command interneurons AVA and AVB). The level 3

neurons appear to control much more precise behaviours, for example SMD reduces the angle of the post reversal turn and thus the frequency of omega turns. SMD and RIV regulate the frequency of omega turns and the AVA command interneurons regulate reversal frequency.

It seems likely that the ethanol conditioning procedure affects this circuit but where? Intoxication causes normal reversals but low omega turns and withdrawal causes low reversals but high omega turns. This does not correlate with either on food (extremely high frequency of short reversals), local search (high reversals and omega turns) or dispersal (low reversals and omega turns) behaviours. It is therefore likely that the effects seen with conditioning are occurring further down the circuit (e.g. level 3). This would make sense as there appears to be two separate effects occurring, an effect on reversals which is not a neuroadaptation effect and an effect on omega turns which is.

The head and neck motor neurones, SMD and RIV, direct omega turns whilst the forward and backward command interneurones control reversals (Gray et al 2005). Intriguingly, laser ablation of the reverse command interneurone AVA resulted in worms that exhibited omega turns in the near complete absence of reversals i.e. unaccompanied omega turns (Gray et al 2005) and thus superficially would appear to phenocopy this aspect of ethanol withdrawal. However, whether or not this laser ablation causes an overall increase in omega turns, as seen for ethanol withdrawal, is not known and it seems unlikely that an increase in omega turns produced through this circuit could be rescued by relief from withdrawal without affecting the rate of reversals. Nonetheless, it is possible that altered signalling through the AVA pathway

could contribute to the increase in frequency of unaccompanied omega turns in ethanol withdrawal.

Further neurones of more interest in this regard are the head motorneurones, SMB, SMD and RIV. Laser ablation of SMB increases the amplitude of dorsal-ventral head turns leading to very loopy movement whilst laser ablation of SMD and RIV has the opposite effect leading to a decrease in omega turns (Gray et al., 2005). A decrease in SMB signalling is therefore very similar to the ethanol withdrawal behaviour, thus in ethanol withdrawal the output from SMB, SMD and RIV may be altered. Whilst the neural basis of unaccompanied omega turns in ethanol withdrawal remains to be defined, the analysis described above highlights the excellent opportunity for a systems level approach provided by defining withdrawal in an animal in which the circuits driving sub-behaviours are relatively simple and delineated.

5.3.6 Withdrawn worms show a loopy behaviour which is relieved by a low dose of ethanol

Withdrawn worms show a significant increase in the frequency of omega turns unaccompanied by a reversal (Figure 5.13) (and therefore a non-significant overall increase in the frequency of omega turns, despite the decrease in reversal frequency – data not shown). They also have an increased likelihood of curling into a ball after a reversal (Figure 5.14). These are both partially relieved by a low dose of ethanol. This is thus likely to be an effect of neuroadaptation to ethanol. Withdrawn worms also appear to have increased amplitude of body bends compared to control worms on visual observation. This is all reflected in the fact that withdrawn worms show

increased loopyness of locomotion, according to video analysis described earlier. This is also partially relieved by a low dose of ethanol (Figure 5.17).

Intoxicated worms have been previously described as having a decreased amplitude of body bends (Davies et al., 2003) and visual observation of intoxicated worms confirms this. However using the video analysis program no significant difference in loopyness can be detected between the control, intoxicated and tolerant worms, although the intoxicated and tolerant worms do appear to have a greater variability in loopyness (Figure 5.17). The rate of reversals and unaccompanied omega turns is also similar between control and intoxicated worms (Figures 5.12 and 5.13). However in both intoxicated and tolerant worms a reduced percentage of their reversals are followed by an omega turn and an increased percentage are followed by no change in direction (Figure 5.14), resulting in an overall non-significant decrease in the frequency of omega turns (data not shown). Part of the reason for the lack of detectable reduction in loopyness may be that a proportion of the intoxicated worms move very little and may remain non-straight line positions for a large period of the video, thus receiving higher than expected measures of loopyness despite low amplitude body bends and few omega turns.

This analysis of loopyness therefore shows that intoxication and withdrawal, despite both reducing the ability of worms to reach the food in a food race, are two very different behaviours. Withdrawal increases overall loopyness, frequency of unaccompanied omega turns and likelihood of curling into ball after a reversal. It also appears to increase the amplitude of body bends. Intoxication doesn't increase loopyness, and it decreases the likelihood of an omega turn after a reversal and

increases the likelihood of no change of direction after a reversal. It also appears, on visual inspection, to decrease the amplitude of body bends. This indicates that ethanol intoxication and withdrawal are distinct, antonymous behaviours. This is what would be expected if withdrawal was the consequence of a neuroadaptation to ethanol's presence revealed by the removal of ethanol.

The increased frequency of omega turns may be a cause of the reduced ability of withdrawn worms to reach the food. It has been shown that worms containing a mutation that results in a constitutively open GLR-1 channel, referred to a 'lurcher' worms, show hyper-reversal behaviour. This behaviour leads to them performing poorly in the food race (Zheng et al., 2004). An increased frequency of omega turns could produce a similar result as both behaviours prevent the worm making long runs in a single direction e.g. towards the food.

It has been shown that a constitutively active form of the small GTP-binding protein RHO-1 results in loopy locomotion in *C. elegans* and that inhibition of RHO-1 function led to very shallow body bends. This was described as being caused by RHO-1 acting to enhance acetylcholine release at the neuromuscular junction (McMullan et al., 2006). Sinusoidal locomotion in *C. elegans* involves muscles on one side of the body being stimulated to contract by cholinergic neurons, which simultaneously stimulate GABAergic neurons to inhibit contraction on the other side of the body. It would thus be interesting to investigate the effect of acetylcholine release, or GABAergic function on intoxication and withdrawal.

5.3.6 Speed and Efficiency are reduced in both intoxication and withdrawal

This study also measured how the interrelated measures of speed and efficiency of worm locomotion were affected by intoxication and withdrawal. Both intoxicated and withdrawn worm show significant decreases in both speed and efficiency of locomotion, although in both cases the response is more extreme in intoxication. The efficiency and speed of worm locomotion in withdrawn worms is partially relieved by a low dose of ethanol. However tolerance does not significantly reduce the effect of intoxication on the speed or efficiency of worm locomotion.

This shows that whilst intoxication and withdrawal are two different behaviours, they both cause a reduction in the ability of the worm to move. This clearly relates to the performance of the worm in the food race. Intoxication shows a more extreme reduction in speed and efficiency. The fact that they both give similar and fairly poor performances in the food race may be caused by the increased frequency of omega turns in the withdrawn worms as discussed earlier.

5.3.7 Summary

C. elegans show neuroadaptation to the chronic presence of ethanol. This can be demonstrated in the food race. Intoxicated worms show a reduction in the ability to reach the food which is improved in tolerant worms. Withdrawn worms show a reduction in the ability to reach the food which is partially relieved by a low concentration of ethanol (relief from withdrawal). These effects are at least partially caused by distinct opposing effects on locomotion.

Chapter 6 - Genetic analysis of the mechanism of neuroadaptation to ethanol

6.1 Introduction

In the previous chapter it was demonstrated that *C. elegans* show neuroadaptation in response to chronic pre-conditioning with ethanol. This was revealed by the degree that worm behaviour was modified in a food race. Intoxicated worms show a reduction in the ability to reach the food. This is less pronounced in worms previously chronically exposed to 250-350mM ethanol indicating that these worms exhibited tolerance (see Figure 5.2). In addition withdrawn worms show a reduction in the ability to reach the food which is relieved by a low concentration of ethanol (relief from withdrawal) (see Figure 5.4). These effects are caused by distinct and opposing effects of intoxication and withdrawal on locomotion (see Figures 5.11, 5.13, 5.17, 5.18 and 5.19).

The aim of this chapter is to investigate the mechanism by which the neuroadaptations highlighted above and described in the previous chapter, occur, by investigating which candidate genes, and therefore proteins, are required in order for it to occur. The mechanism of neuroadaptation can be investigated by forward or reverse genetics.

First of all, as described in Appendix A, a forward genetic screen was performed for mutants defective in withdrawal behaviour. The screen used criteria for selection in which worms undergoing withdrawal which had reached the food fifty minutes into the food race, a time point at which wild type withdrawn worms would not be expected to have done so, would be selected for analysis. The basis for these criteria is described in Appendix A. However this produced no strains of worms defective in

withdrawal behaviour. One reason for this could have been because the screen was not saturated due to a high time requirement per genome screened. Another reason could be because the withdrawal behaviour was a result of slight changes in many different pathways controlling behaviour and thus no individual mutant showed sufficiently different withdrawal behaviour to be detected by the screen. Alternatively the criteria used to identify the mutants (the food race) may not have allowed for detection of mutants that were also impaired in locomotion.

A candidate gene approach was therefore pursued. As described in the introduction (see section 1.4), a wealth of literature pinpoints the regulators implicated in the response to ethanol in mammalian systems at concentrations relevant to human alcohol dependence. In the worm genetic perturbation of many of the major neurotransmitter pathways implicated in the ethanol response is possible without lethality (Brenner, 1974). In addition some genes have been previously identified as being involved in the responses to acute and chronic ethanol in *C. elegans*. The candidate genes described below were selected for investigation.

Gene	**Protein encoded**	**Strain**	**Allele**	**Predicted effect**	**Phenotypes**
slo-1	The main pore-forming subunit of the BK potassium channel	N1968	*js379*	Null mutation	Jerky locomotion, aldicarb hypersensitive Emodepside resistant
		XA3747	*pd23*	Loss of function (lof) mutation	Jerky locomotion, Emodepside resistant
		XA3748	*pd24*	Loss of function (lof) mutation	Jerky locomotion, Emodepside resistant
	Rationale: Worms with lof mutations in this gene have been reported to be resistant to the acute effects of ethanol.				
	References: (Davies et al., 2003;Guest et al., 2007)				

Gene	Protein encoded	Strain	Allele	Predicted effect	Phenotypes
npr-1	The NPY receptor-like neuropeptide receptor	AX201	*ky13*	Null mutation	Social feeding, altered locomotion
		CB4856	Hawaiian strain	Reduced function version	Social feeding, altered locomotion
	Rationale: This gene has been reported to be involved in the development of acute tolerance to ethanol and in a putative ethanol-withdrawal behaviour. In addition NPY is implicated in the chronic response to ethanol in mammalian systems.				
	References: (de Bono and Bargmann, 1998;Davies et al., 2004a;Thorsell, 2007)				
egl-3	A *C. elegans* homolog of a mammalian proprotein convertase that participates in peptide precursor processing	XA3741	*ok979*	Almost total absence of neuropeptides in the worm with one peptide detected out of 75 in one study.	Egg-laying defective, coiler
	Rationale: Many different peptides have been implicated in the development of ethanol dependence in mammals including CRF, NPY and the opioid peptides.				
	References: (Husson et al., 2006;Li and Kim, 2008;Koob et al., 1998)				
ACh	The effect of acetylcholine signalling will be investigated using a pharmacological assay.				
	Rationale: ACh signalling has previously been shown to influence the loopyness of body bends in a similar manner to ethanol withdrawal.				
	References: (McMullan et al., 2006)				
unc-25	The GABA biosynthetic enzyme glutamic acid decarboxylase	CB156	*e156*	Loss of function allele leading to reduced GABA levels	Shrinker, uncoordinated
unc-49	This gene has multiple splice variants which each encode different subunits of a heteromeric $GABA_A$ receptor.	CB407	*e407*	Null mutation in one of the subunits of this receptor (UNC-49B), which is required to form functional GABA receptors at the neuromuscular junction in body wall muscles.	Shrinker, uncoordinated
	Rationale: GABA and ACh act antagonistically to produce normal sinusoidal locomotion and thus it was thought that ethanol might affect the amplitude of body bends by an action on GABAergic signalling based on the observations in the previous chapter (see Figure 5.11). In addition GABAergic signalling has been implicated in the effects of ethanol in both mammalian and other invertebrate systems				
	References: (Enoch, 2008;McIntire et al., 1993;Chalfie and White, 1988)				

Gene	Protein encoded	Strain	Allele	Predicted effect	Phenotypes
cat-2	Tyrosine hydroxylase an enzyme required for dopamine synthesis.	CB1112	*e1112*	Nonsense mutation, leading to depleted dopamine levels	Altered foraging behaviour
eat-4	An ortholog of the mammalian BNPI vesicular glutamate transporter	MT6308	*ky5*	Loss of function allele which results in severely reduced glutamate signalling	Altered foraging behaviour, defective pharyngeal pumping, altered chemotaxis to NaCl.
	Rationale: Dopaminergic and glutamatergic signalling have been implicated in the control of reversals and high angled turns in *C. elegans.* In chapter 5 it was shown that ethanol conditioning affects the rates of reversals and omega turns. Therefore the question of whether mutations in dopaminergic or glutamatergic signalling affected ethanol conditioning was investigated.				
	References: (Hills et al., 2004;Lee et al., 1999)				
tph-1	Tryptophan hydroxylase, the enzyme that encodes the rate limiting step in 5-HT biosynthesis.	GR1321	*mg280*	Loss of function leading to severely reduced 5-HT levels.	Reduced egg laying, pharyngeal pumping, increased lifespan
	Rationale: 5-HT signalling has been implicated as being very important in the development of ethanol dependence in mammalian systems.				
	References: (Koob et al., 1998)				

Table 6.1 Summary of candidate genes and the *C. elegans* strains used to investigate them in the following chapter. Strain details from http://www.wormbase.org. lof = Loss of function.

In the previous chapter two main experimental procedures were used to investigate the development of neuroadaptation in the worm. These were the food race assay and video analysis of the movement of a worm on a food race plate. The movement of the worm was recorded for video analysis five minutes after being added to the food race plate, at a time point that may reflect a local search state (see section 5.1). Three parameters, loopyness, efficiency and speed were measured (see sections 2.9.1.4-2.9.1.7 for definitions). The alterations seen in these parameters are reflected in the differing ability of the worms to reach the food in the food race. Comparison of the alterations in these parameters demonstrated that withdrawal was a different behaviour to intoxication (see Chapter 5). It was shown that both intoxication and

withdrawal decrease the speed and efficiency of worm locomotion, although intoxication has the greater effect. However withdrawal also produces an increase in the loopyness of locomotion, whereas intoxication causes its effects without increasing the loopyness of locomotion, indicating that these behaviours are distinct.

These parameters will be briefly summarised. The loopyness of the worm's shape in a frame can be calculated as the mean perpendicular distance of the ten node centres from a linear regression line drawn between them (see section 2.10.2 and Figure 6.1A). The mean loopyness of each worm in each video is then plotted. The efficiency of worm locomotion can be described by dividing the distance travelled by the centre of mass of the worm by the distance of the sinusoidal path that the worm actually covers (see section 2.10.2 and Figure 6.1B). The speed of the worm on plates is calculated by the distance travelled by its centre of mass over time (see section 2.10.2).

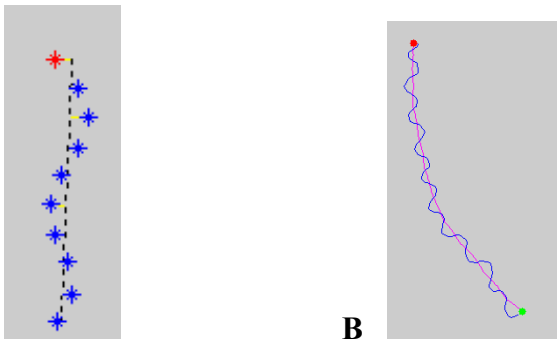


Figure 6.1 Illustration of loopyness and efficiency A. Illustration of the linear regression line drawn between ten node centres along the length of a worm which is used to calculate loopyness as described in section 2.10.2 B. Illustration of the sinusoidal path the worm actually covers (blue) compared to the distance travelled by its centre of mass (pink). This is used to calculate efficiency.

In this chapter the behaviour of the mutant strains listed above was investigated in response to intoxication and withdrawal. In addition the extent of relief from withdrawal in response to a low dose of ethanol, and the development of tolerance to

intoxication were investigated. As many of the strains under investigation have phenotypes that include some locomotion defects the majority of the strains were investigated using the video analysis procedure. The videos were taken at the five minute time point as this had been previously shown to be a time point where statistically significant alterations in unaccompanied omega turns occurred.

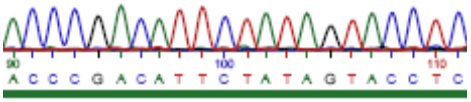
This approach based on a comparison of untreated and variously ethanol treated worms circumvents the limitation that mutants may exhibit extreme impairment in food race capability, which may prevent them reaching the food in the time course of the assay, or at all. Furthermore a comparison of mutant and wild type controls was routinely run which allowed identification of mutants which were phenocopying withdrawal and intoxication phenotypes seen in ethanol treated wild-type worms.

6.2 Results

6.2.1 The strain *slo-1 js379* does not appear resistant to acute ethanol in the thrashing, body bends and food race assays.

The strain *slo-1 js379* contains a stop codon prior to the pore region in the main pore forming subunit of the BK potassium channel (see Figure 6.3). This is therefore likely to be a null mutation. The genotype of the *slo-1 js379* strain was confirmed by sequencing (see Figure 6.2).

Part of *js379* sequence produced from sequencing



*

ACCCGACATTCTATAGTACCTC

Part of sequence for the *slo-1* gene in wild type

caacaaaattcaaatttctcagaacccagctgatatgggggtcattttgatgcttacaaact

cagaaatcatcttaaaatcgcacggataactatttttgaataactatcgaaaaaaaattcaa

gttttaaaaatttcaaaaactcaaaaaattccagGATTCCGTTTCCTCCGTGCTCTTCGCCT

CATGACCGTACCCGACATTCTACAGTACCTCAACATCCTGAAAACATCTTCATCAATCCGAT

TGACACAGTTGGTCACAATTTTCGTGGCGGTTTGTCTGAC

Figure 6.2 The DNA sequence of the region of *slo-1* encompassing the predicted mutation in the allele *js379*. These results represent the read from a genomic sequence reaction of DNA extracted from *slo-1 js379* worms and show that the C→T point mutation is present as expected. The mutation is highlighted blue. The area surrounding the mutation is highlighted pink. The sequencing primer is highlighted yellow. The start point of the sequence produced from sequencing reaction is highlighted green. Capitalised letters highlight exon sequence.

Strain	Mutation	Location/ effect of mutation	Ethanol resistance	In paper?
eg7	E286>K	Affects an absolutely conserved amino acid in extracellular face of SLO-1	Resistant	Davies et al.
eg73 md1715	G289>E	Mutation on extracellular face of SLO-1. Functionally inactive in oocytes.	Resistant	Davies et al. and Wang et al.
eg24	G841>R	Affects an absolutely conserved amino acid in cytoplasmic tail of SLO-1	Resistant	Davies et al.
eg142	W46>STOP	Stop codon is early in the first transmembrane domain therefore likely to be null	Resistant	Davies et al.
js118	Deletion/ frameshift	Channel truncated prior to calcium bowl (after S9). Functionally inactive in oocytes.	Resistant	Davies et al. and Wang et al.
js379	Q251>STOP	Stop codon prior to pore region (S4) therefore likely to be null	Not resistant/ Resistant	Wang et al., Wu et al. and this study
md1745	Q134>STOP	Stop codon prior to pore region (between S0 and S1) therefore likely to be null	Unknown	Wang et al.
js380	W850>STOP	Channel truncated prior to calcium bowl (after S9)	Unknown	Wang et al.
js381	Q914>STOP	Channel truncated prior to calcium bowl (after S9)	Unknown	Wang et al.

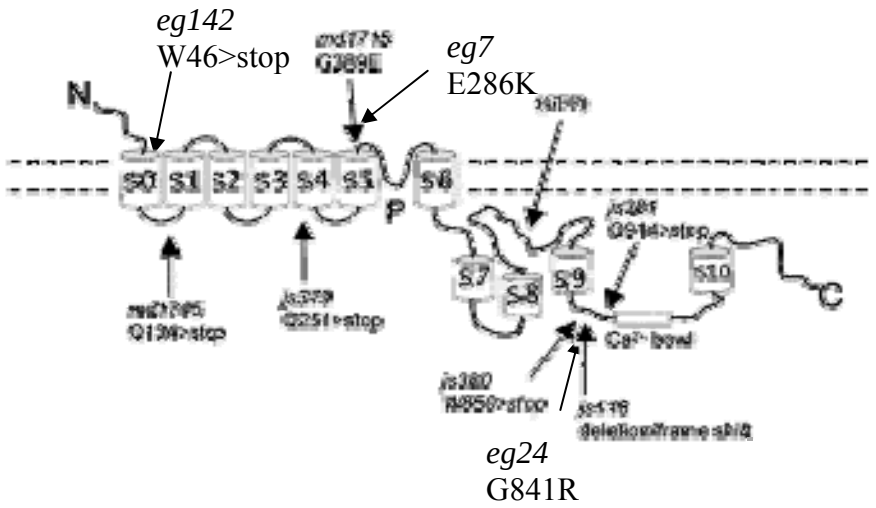


Figure 6.3 Location of the mutations in various alleles of *slo-1*. Image adapted from Wang et al. (Wang et al., 2001;Davies et al., 2003)

As described in Chapter 3, immersion of N2 *C. elegans* in ethanol (range 100–500mM) inhibited, but did not completely abolish, thrashing behaviour (Figure 3.1). This effect is concentration-dependent and half-maximal at approximately 300mM (Figure 3.1). Notably, at each concentration, inhibition reaches a steady-state value within 5 min (Figure 3.2). This effect is also seen in *slo-1 js379* worms (Figure 6.4).

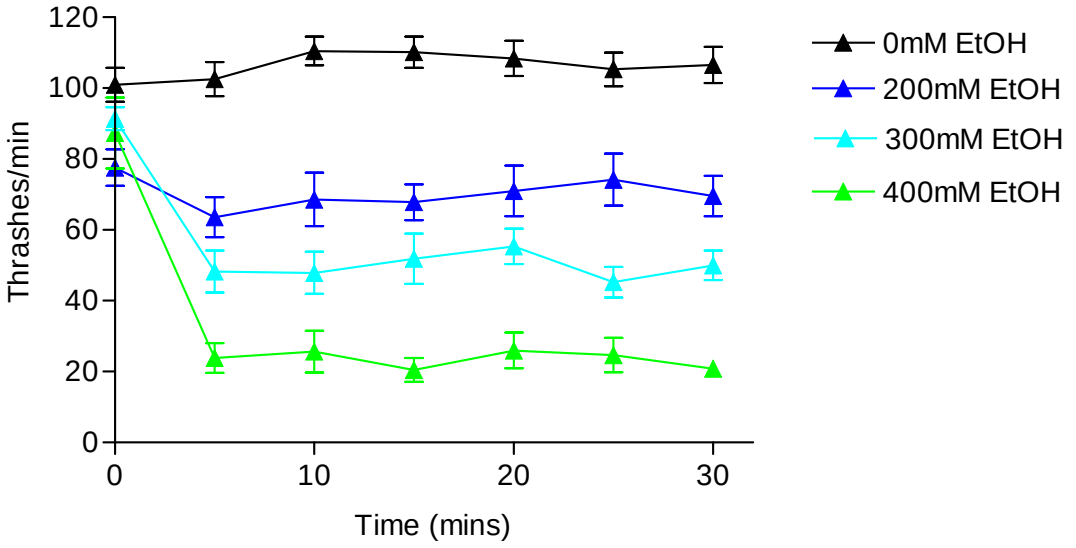


Figure 6.4 Time course for the inhibitory effect of ethanol on *slo-1 js379* worms in the thrashing assay. The worm reaches a steady rate of thrashing before the first time point at 5 min. The zero time point shows the thrashing rate of the worm before the addition of ethanol. Each worm was tested at all time points of one concentration. Results are the mean ±s.e. of at least six independent worms.

Figure 6.5 shows a comparison between the behaviour of N2 and *slo-1 js379* in the same conditions at 400mM ethanol. There is no significant difference between the two genotypes ($F_{1,176}$=1.102, P=0.309), therefore *slo-1 js379* worms do not appear to be resistant to ethanol when assessed in the thrashing assay.

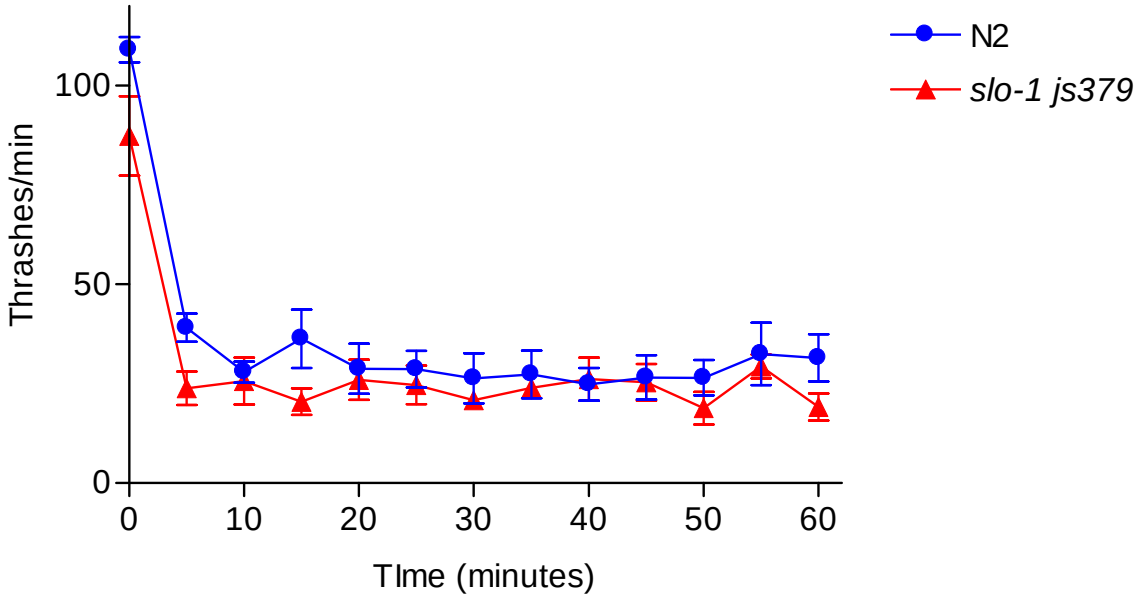


Figure 6.5 Thrash rate in response to 400mM ethanol for N2 and *slo-1 js379* worms. The ethanol is added immediately after the initial 0 min reading. Results are the mean ±s.e. of at least six independent worms.

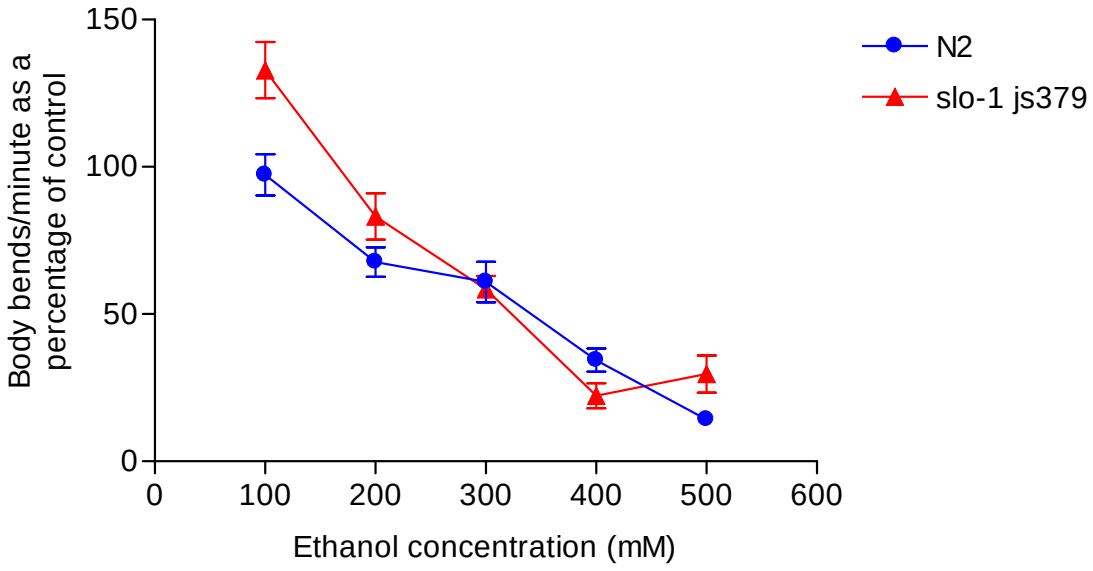


Figure 6.6 Rate of body bends in ethanol as a percentage of basal rates of body bends for N2 and *slo-1 js379* worms. Results are the mean ±s.e. of at least ten independent worms. Mean rate of body bends of control worms was 51.58/min for N2 and 51.60/min for *slo-1 js379*

Figure 6.6 shows that in the body bends assay *slo-1 js379* worms are not resistant to high and medium ethanol concentrations (from 200mM to 500mM a two way ANOVA shows no significant effect of genotype ($F_{1,165}$=2.029, P=0.156)), but they

could be hyperactive in the presence of low concentrations of ethanol. However this potential hyperactivity is not seen in locomotion on agar (see Figure 6.7).

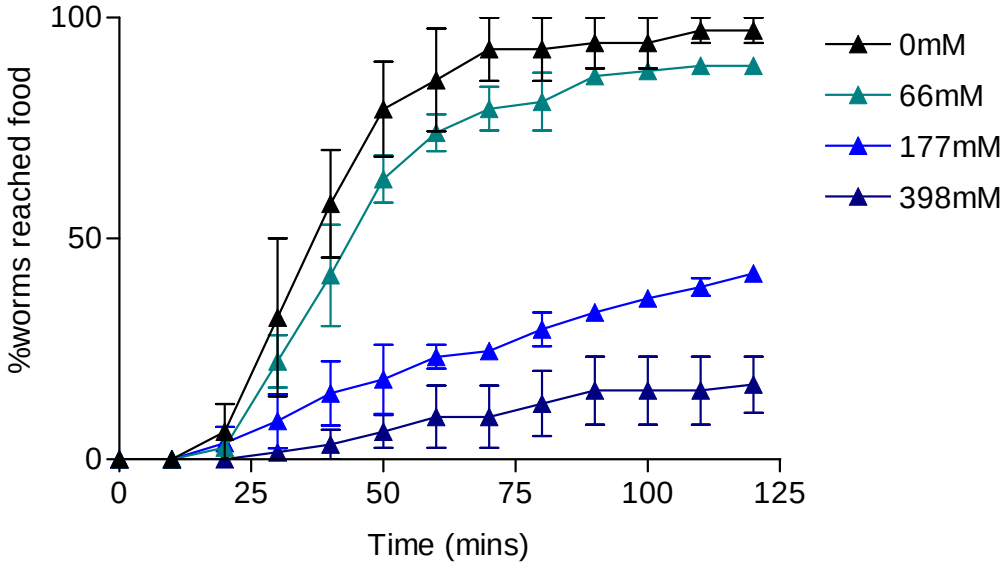


Figure 6.7 Effect of three acute concentrations of ethanol on the percentage of *slo-1 js379* worms reaching the food over a two hour period. Each point is the mean ±s.e. of two food race assays

In the food race assay (Figure 6.7) acute ethanol at concentrations of 177 or 398mM significantly interferes with the ability of *slo-1 js379* worms to reach the food. This is a similar effect to that seen in N2 worms. This assay together with the thrashing and body bends assays appears to indicate that *slo-1 js379* worms are not resistant to acute ethanol.

6.2.2 Lack of resistance to ethanol is not a strain specific effect.

The lack of resistance to ethanol of *slo-1 js379* worms described above contradicts previously published results (Davies et al., 2003), in which, as previously described, multiple alleles of *slo-1* came out of a screen for resistance to ethanol. The strain used in the experiments above was not one of those that came out of this screen although it has recently been described as ethanol resistant by another group (Wu et al., 2008).

The *js379* allele of *slo-1* has a single base C to T mutation which inserts a stop codon into the fourth transmembrane domain, which is before the pore region (Figure 6.3). This is thus a presumed null. Both *md1745,* which contains a stop codon between transmembrane domains zero and one and is thus a presumed null, and *js118,* which contains a frameshift mutation in the C-terminal domain, were identified as ethanol resistant in the screen. As *js379* is a presumed null and as other presumed nulls and milder mutations of the gene have been shown to be resistant, *js379* would normally be presumed to share this phenotype.

To investigate if this lack of resistance was a strain dependent effect the response to acute ethanol of two other strains with mutations in *slo-1* was examined. Both the *slo-1 pd24* and *slo-1 pd23* alleles have mutations in the RCK domains of SLO-1. These are therefore not necessarily null mutations. They were isolated in a screen for worms that were resistant to the anthelmintic drug emodepside, along with other worms containing loss of function mutations in *slo-1*, and they have been shown to complement *slo-1 js379* in this phenotype. This indicates that they are likely to produce at least a reduction of function in *slo-1*. They have also been shown to exhibit a similar locomotion phenotype to *slo-1 js379* consisting of an increased frequency of reversal behaviour (Guest et al., 2007).

The behaviour of these strains was tested in response to acute ethanol in the thrashing assay by Amanda Pugh (School of Biological Sciences, University of Southampton). The *slo-1 pd23* worms had a thrash rate in 400mM ethanol of 22% of basal compared to a rate of 23% of basal for the matched N2 controls (n=10). This experiment was repeated with *slo-1 pd24* worms which had a thrash rate of 26% of basal compared to

26% of basal for the N2 controls (n=20). Neither strain therefore showed noticeably different behaviour to wild type in response to acute ethanol in this assay. This would indicate that the lack of resistance to ethanol is not a strain specific effect of the *slo-1 js379* strain.

6.2.3 The response of *slo-1* mutants to ethanol conditioning

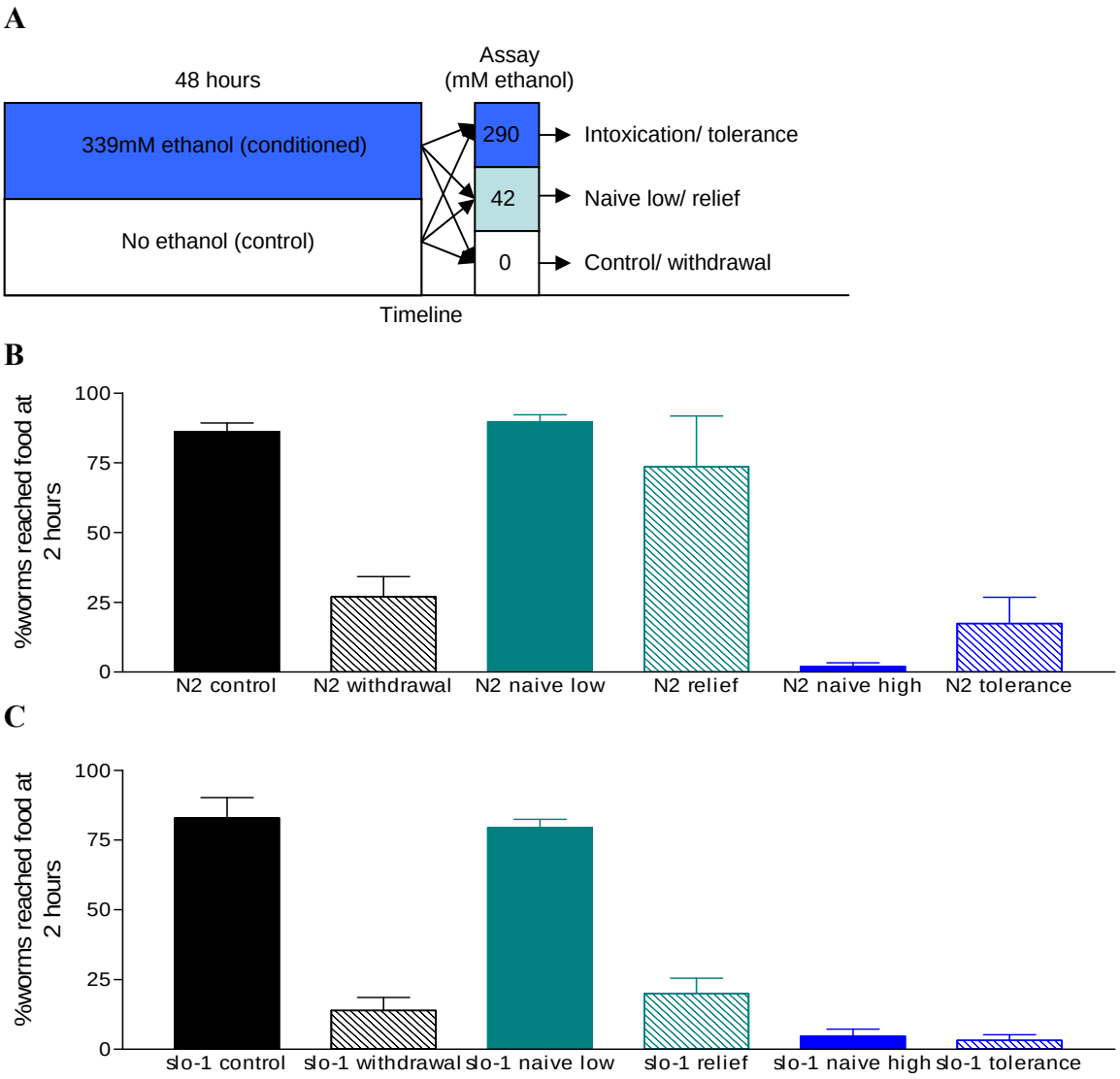


Figure 6.8 (A) Timeline of the experiment. Effect of 48 hours conditioning with 339mM ethanol on the percentage of (B) N2 and (C) *slo-1 js379* worms have reached the food after two hours. Each bar is the mean ±s.e. of four food race assays. Filled bars indicate worms naive to ethanol (control, naive low, naive high/intoxication), striped bars indicate worms pre-exposed to ethanol (withdrawal, relief, tolerance). Black bars are assayed in the absence of ethanol (control, withdrawal), green bars at 42mM ethanol (naive low, relief) and blue bars at 290mM ethanol (naive high/intoxication, tolerance).

In the food race assay (Figure 6.8C) *slo-1 js379* worms show a reduced ability to reach the food when acutely exposed to a 290mM ethanol (naive high/intoxication). This agrees with previous data (Figure 6.7). They also show a reduced ability to reach the food when conditioned on 339mM ethanol for 48 hours and then removed entirely from ethanol (withdrawal). This is a similar effect to that seen in matched N2 controls (Figure 6.8B). However the withdrawal behaviour in the *slo-1* worms is not relieved by a low dose of ethanol (relief from withdrawal Figure 6.8 B and C) as it is in N2. The development of tolerance, seen in the N2 worms (Figure 6.8B) as an improvement in the ability of worms to reach the food at high concentrations of ethanol after chronic exposure, is also not evident in the *slo-1* worms (Figure 6.8C). This may indicate that *slo-1 js379* worms do not undergo neuroadaptation to ethanol in the same manner as N2 worms.

In order to investigate this further, the rate of recovery from withdrawal in N2 and *slo-1 js379* worms was examined (Figure 6.9). It was considered that if *slo-1* worms were not undergoing neuroadaptation to ethanol, but were still impaired in their performance after conditioning with ethanol (*slo-1* withdrawal in Figure 6.8C), then this impairment might be due to a permanent toxic effect of the ethanol conditioning and/or an indirect ethanol induced adaptive response (e.g. from reduced feeding). In the first case this could be investigated by measuring whether they recovered from ethanol conditioning to this same extent as N2 worms. However, over a 24 hour period *slo-1 js379* appeared to recover from withdrawal at a similar rate to N2 (Figure 6.9).

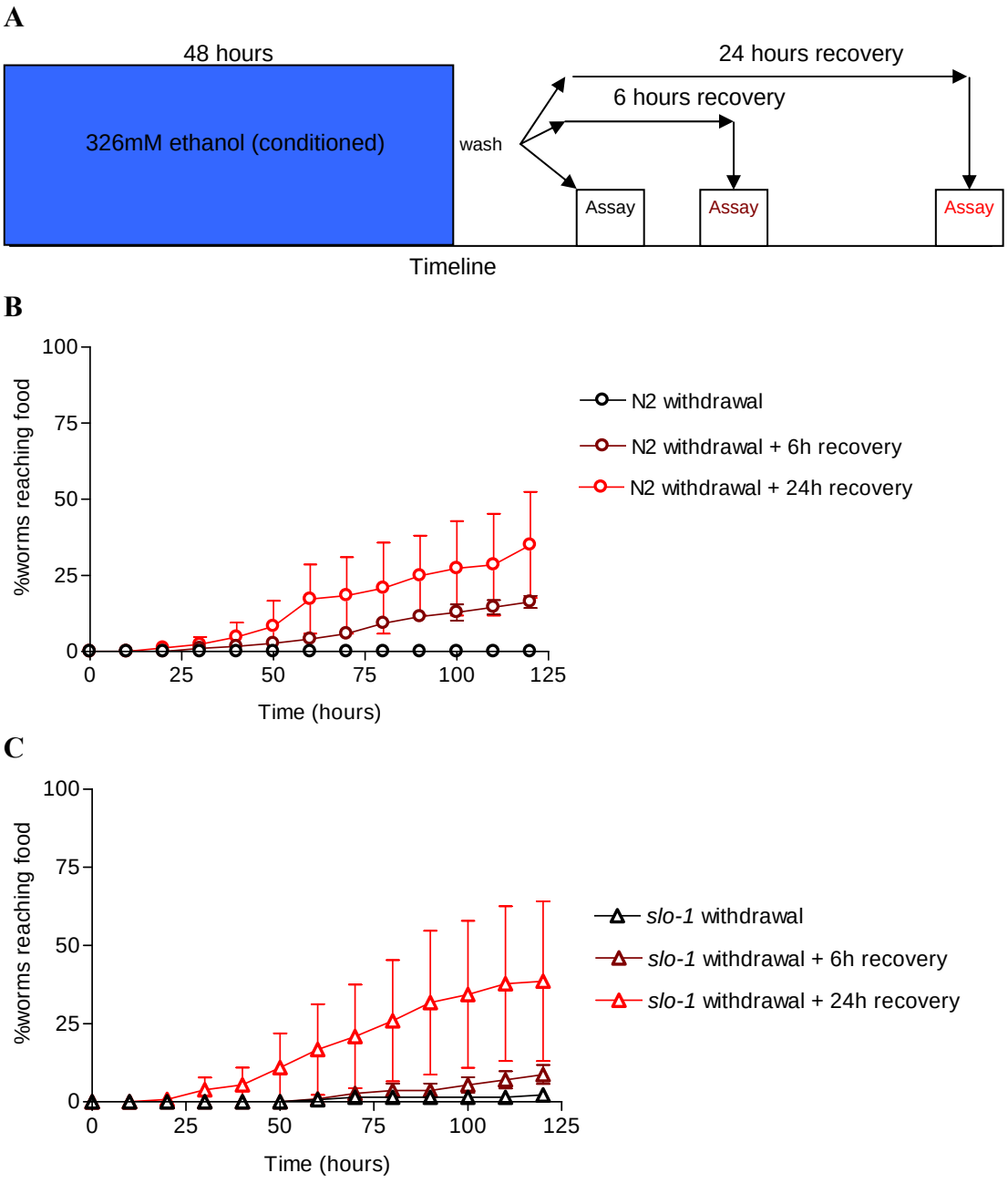


Figure 6.9 Recovery from conditioning. (A) Timeline of the experiment. (B) N2 or (C) *slo-1 js379* worms conditioned at 326mM for 48 hours then tested in the food race in the absence of ethanol either immediately or after either 6 or 24 hours of recovery on non-ethanol food plates. Each point is the mean ±s.e. of two food race assays.

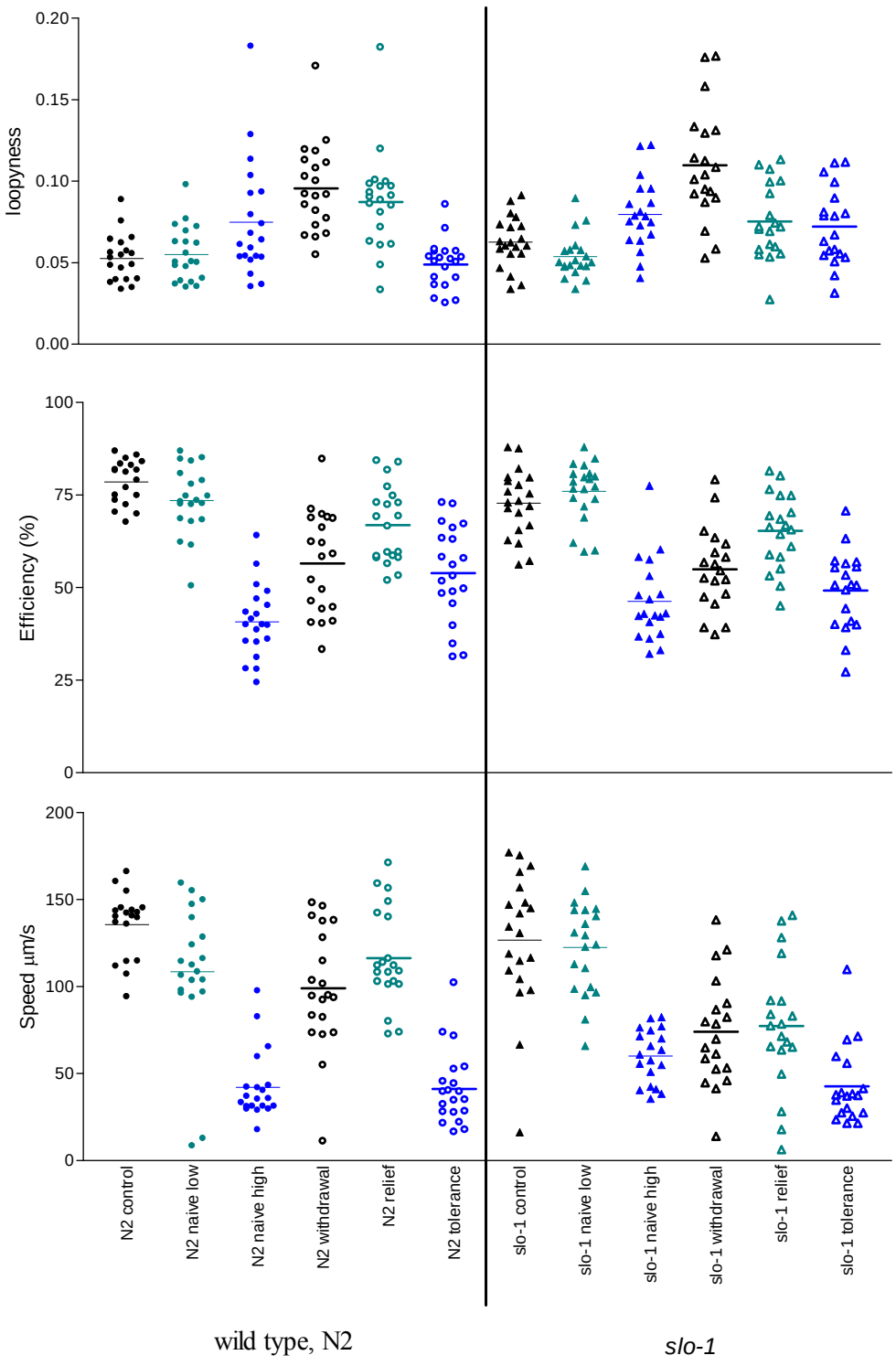


Figure 6.10 *slo-1 (js379)* responds like wild-type to acute and chronic ethanol exposure. The data for wild-type controls are shown as circles and for *slo-1* as triangles. These data were obtained using automated off-line analysis of videos collected as described in chapter 5. Three measurements of motility were made: A, 'loopyness' which provides a readout of the difference between the worms posture and a straight line; B, 'efficiency' which provides an indication of the translation of the overall movement of the animal into its trajectory and C 'speed', defined as distance travelled per unit time where distance was the measured as a straight line from the start to end point position of the animal. Each data point represents a measurement from a single worm and the bars indicate the mean for each data set. See Appendix B for statistical analysis.

The response of *slo-1 js379* worms to intoxication, tolerance, withdrawal and relief from withdrawal was then observed using video analysis to provide a measurement for loopyness, efficiency and speed as previously described in the Chapter 5 (Figure 6.10). The N2 matched controls showed an increase in loopyness and a decrease in efficiency and speed as expected in response to withdrawal. They also showed the expected greater decrease in efficiency and speed without a change in loopyness in response to intoxication. This was the response expected as it agreed with the results described in Chapter 5. The *slo-1 js379* worms showed significant effects of intoxication and withdrawal in the same manner as the N2 worms. They also showed a significant effect of relief from withdrawal on both loopyness and efficiency, indicating that they can undergo neuroadaptation to ethanol. This contradicts the data in the conditioned food race experiment (Figure 6.8).

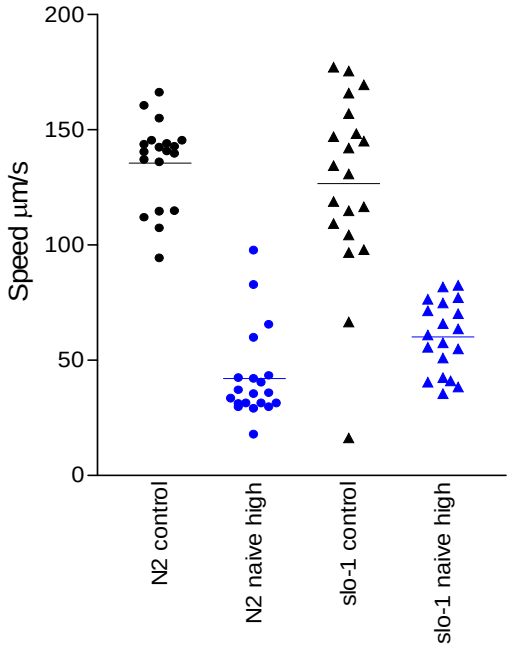


Figure 6.11 Direct comparison of the speed of *slo-1 js379* and N2 worms in response to intoxication

One interesting point is the speed of the *slo-1 js379* worms in response to intoxication is decreased significantly less than N2 worms (t_{37}=3.174) (Figure 6.11). This is interesting as *slo-1* worms have been reported to be resistant to the effects of acute ethanol when speed on plates was measured (Davies et al., 2003), but previous experiments in this study had not shown *slo-1* worms to have any resistance to the effects of acute ethanol when the related parameters of rate of thrashing, rate of body bends and rate of reaching food in the food race were measured (Figure 6.4, 6.5, 6.6 and 6.7).

6.2.4 The neuropeptide receptor NPR-1

Comparison of the Hawaiian strain (CB4856) of *C. elegans* with the Bristol strain (N2) provides an insight into an important class of neuropeptide signalling. The Hawaiian strain is an alternative natural isolate which has a number of single nucleotide polymorphisms (SNPs) when compared to N2. One of these is that the Hawaiian strain of *C. elegans* has been shown to have a lower function 215F allele of the *npr-1* gene, compared to the higher function 215V allele found in the Bristol strain N2 (de Bono and Bargmann, 1998). The Hawaiian strain has been demonstrated to gain an acute (within session) tolerance to ethanol faster than the N2 strain (Davies et al., 2004a).

Thus the response of the Hawaiian strain was investigated in the thrashing assay. If the Hawaiian strain were to gain acute tolerance to ethanol faster than N2, it would be expected that the Hawaiian strain worms would increase their thrashing rate over time during exposure to ethanol.

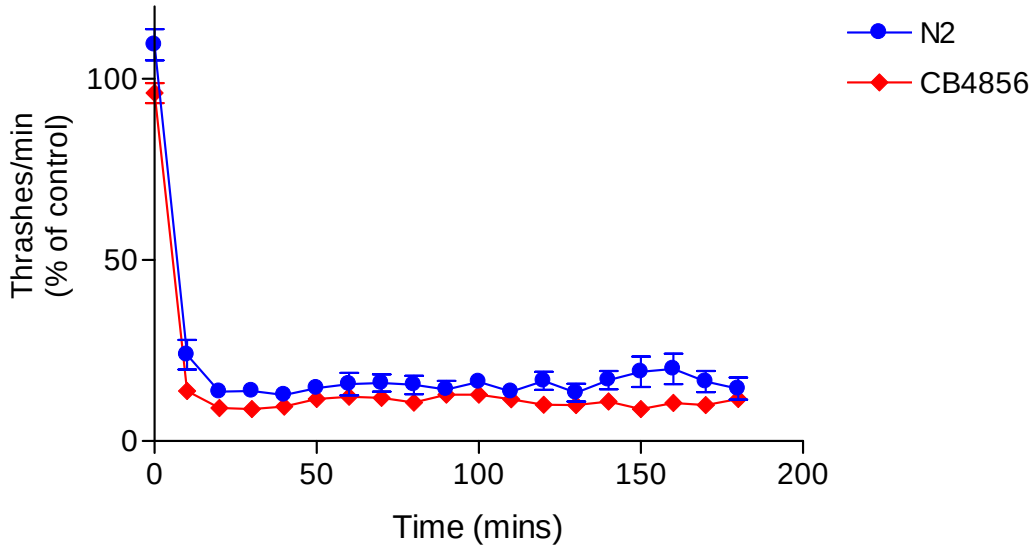


Figure 6.12 Thrash rate in response to 500mM ethanol as a percentage of thrash rate in Dents saline for wild type N2 (Bristol strain) worms which have the higher function 215V allele of the gene *npr-1*, and for CB4856 (Hawaiian strain) worms, which have the lower function 215F allele, over a three hour period. The ethanol is added immediately after the initial 0 min reading. Each point is the mean ±s.e. of at least 11 independent worms.

However these results show that, after reaching a steady behavioural state after the addition of ethanol, neither N2 nor CB4856 show any change in their behaviour over time in the thrashing assay (Figure 6.12).

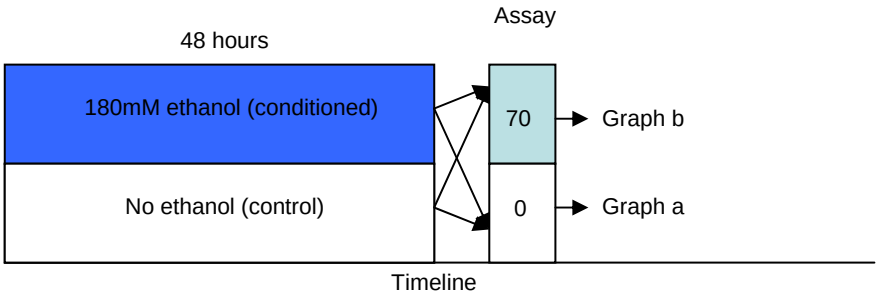
To independently investigate the proposed role of NPR-1 signalling, the response of *npr-1 ky13* mutants to ethanol conditioning was also investigated. This strain contains the nonsense mutation Q61>STOP in *npr-1*, which means that it is a null mutation. This is therefore likely to have a more pronounced phenotype than the Hawaiian strain which only contains a lower function version of the protein encoded by the gene, which causes a reduced level of signalling.

As described in the introduction (section 1.11.4) it has been reported that after 18-22 hours exposure to 350mM ethanol, N2 animals when withdrawn from the ethanol

show a tendency to display clumping and bordering activity, which is a phenotype of *npr-1* mutants (Davies et al., 2004a). This led to the hypothesis that ethanol activated the NPR-1 pathway, causing a consequent downregulation of the pathway over time which was revealed when ethanol was removed.

This could confound our analysis of ethanol conditioning in the food race if ethanol was impacting on foraging and food sensing behaviours. If this was the case and ethanol withdrawal was also phenocopying a deficiency in NPR-1 signalling it would be expected that naive *npr-1 ky13* worms would be unable to reach the food in the absence of ethanol.

A



B

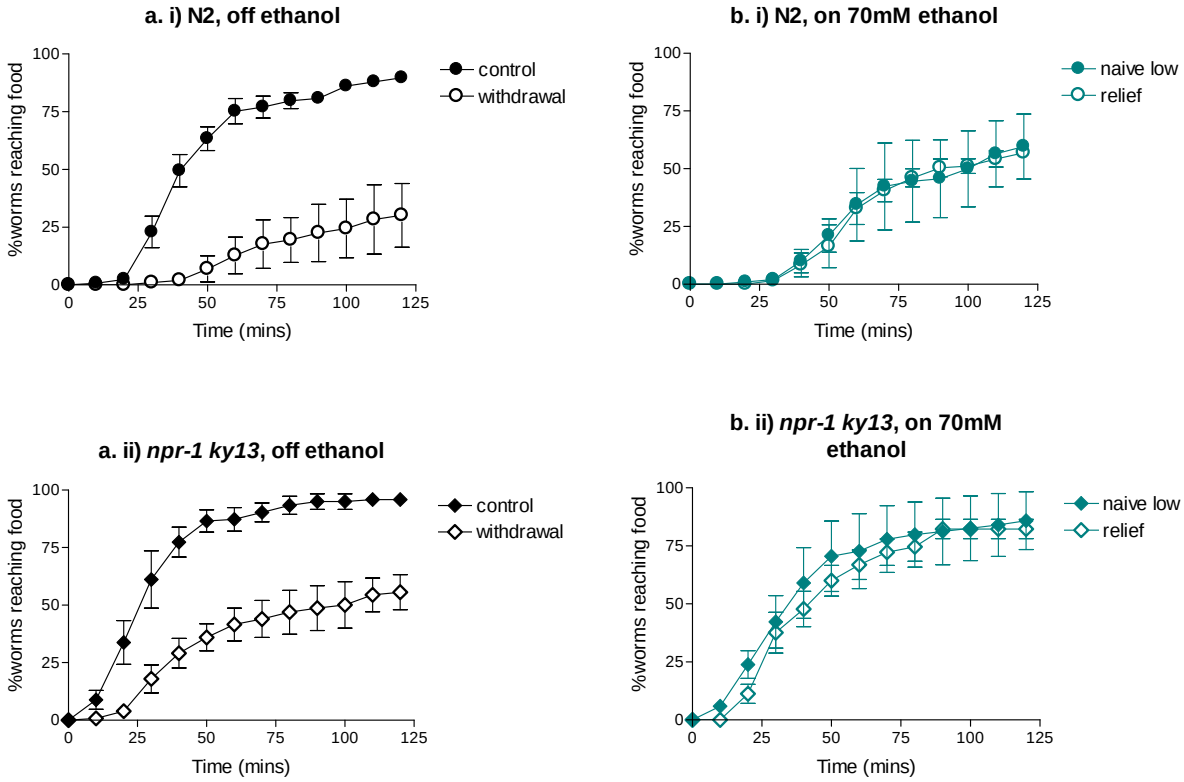


Figure 6.13 (A) Timeline of the experiment (B) Effect of 48 hours conditioning with 180mM ethanol on the percentage of (i) N2 and (ii) *npr-1 ky13* worms reaching the food over a two hour period. The food race was performed off ethanol (a) and in the presence of 70mM ethanol (b). Conditioned worms are indicated by open circles and naive worms by open circles. Each point is the mean ±s.e. of four food race assays.

Accordingly the food race assay was used to analyse the *ky13* worms. The results show that *ky13* worms are capable of reaching the food in the absence of ethanol; in fact they reach the food faster than the N2 worms (Figure 6.13). This is probably explained by the fact that one of the phenotypes of *npr-1* null worms is faster movement on agar plates (de Bono and Bargmann, 1998). The conditioned *ky13*

worms also reach the food faster than the conditioned N2 worms. However the overall pattern of withdrawal and withdrawal relief is maintained, as conditioned *ky13* worms reach the food less quickly than naive worms in the absence of ethanol, and these conditioned worms reach the food faster in the presence of low concentrations of ethanol. These concentrations of ethanol do not affect the naive *ky13* worms. This is shown by a three-way ANOVA in which there is a significant effect of genotype alone ($F_{1,27}$=11.446, P=0.003) and a significant effect of conditioning alone ($F_{1,27}$=18.598, P<0.001), but no significant interaction between genotype and either conditioning ($F_{1,27}$=0.565, P=0.461), acute ethanol concentration ($F_{1,27}$=0.661, P=0.426) or both ($F_{1,27}$=0.669, P=0.423).

The *npr-1 ky13* worms are not affected differently to N2 by ethanol conditioning or acute ethanol, but are faster in the food race assay under all of the conditions shown here.

6.2.5 The effect of ethanol on acetylcholine (ACh) release

Acetylcholine is the main excitatory neurotransmitter at the *C. elegans* neuromuscular junction. Mutations that enhance acetylcholine release have been previously described as causing loopy behaviour in *C. elegans* (McMullan et al., 2006). Acetylcholine release is often inferred by measurement of the time taken to inhibit locomotion in the presence of the acetylcholinesterase (AChE) inhibitor aldicarb. Aldicarb prolongs the presence of ACh in the synaptic cleft thereby causing paralysis through hypercontraction. The aldicarb assay relies on increased synaptic release driving the worm to paralysis (Miller et al., 1996). If the release of ACh from the neuromuscular junction is increased, the worm becomes more sensitive to aldicarb; likewise if it is decreased the worm becomes aldicarb resistant. The rate of paralysis by aldicarb was

therefore measured under the conditions of ethanol intoxication and ethanol withdrawal. Paralysis was defined as the worm not making any movement forwards or backwards in response to nose touch.

Therefore if the loopy behaviour of the withdrawn worms is caused by increased ACh release, increased sensitivity to aldicarb in the withdrawn worms would be expected. Likewise the flatter body bends of the intoxicated worms could be related to decreased ACh release; in which case resistance to aldicarb in intoxicated worms would be expected.

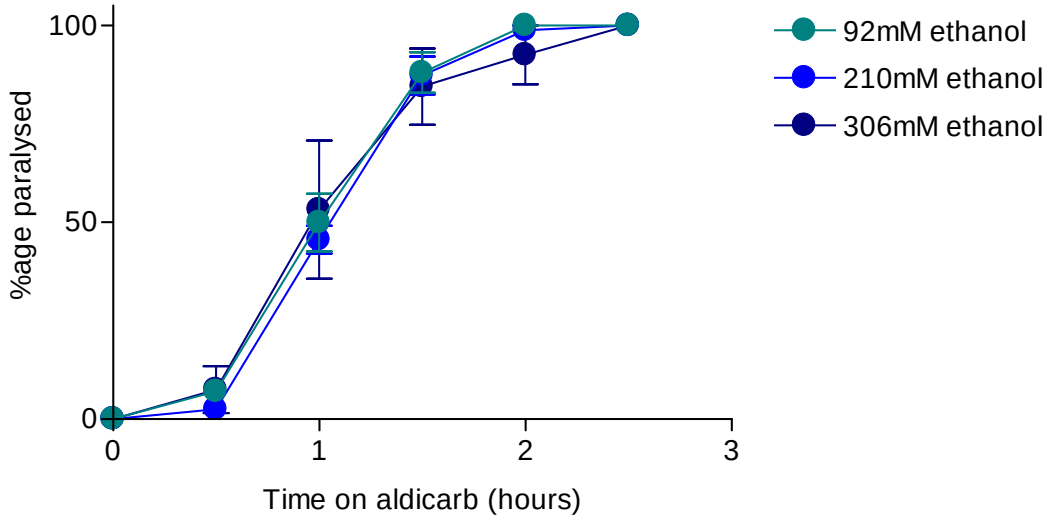


Figure 6.14 Effect of increasing acute concentrations of ethanol in the aldicarb assay. Each point is the mean ±s.e. of at least four plates of 20 worms. Vehicle (ethanol) controls showed no paralysis.

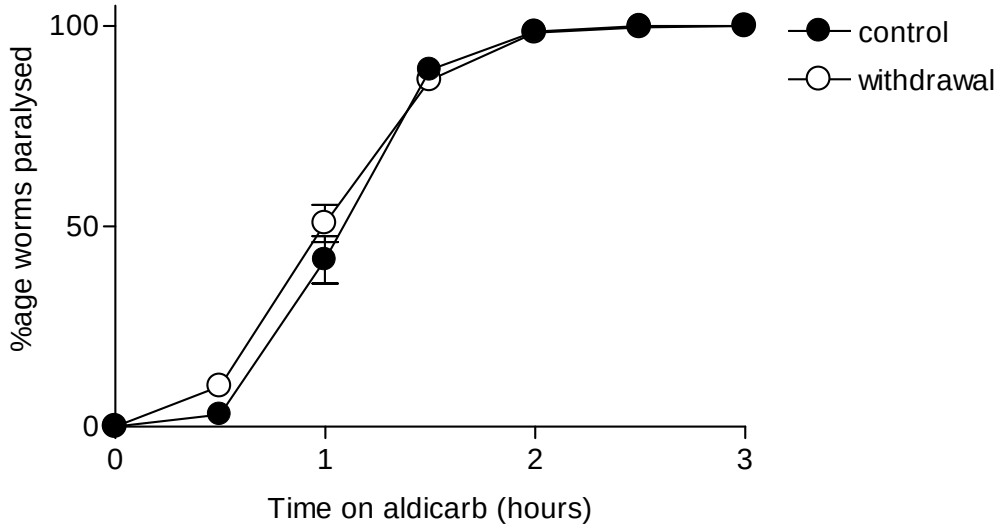


Figure 6.15 Effect of ethanol withdrawal in the aldicarb assay. Withdrawn worms have been conditioned at 246mM ethanol for 48 hours. Each point is the mean ±s.e. of twelve plates of 20 worms. Vehicle (DMSO/ DMSO and withdrawal) controls showed no paralysis.

However neither ethanol withdrawal (Figure 6.15) nor intoxication (Figure 6.14) affected the sensitivity of *C. elegans* to aldicarb. This indicates that neither ethanol withdrawal nor intoxication affects acetylcholine release as measured using the aldicarb assay.

6.2.6 The role of neuropeptides in the development of ethanol dependence

A number of neuropeptides and peptide hormones have been implicated in the development of ethanol dependence in mammals (see Chapter 1). The involvement of neuropeptides in the development of withdrawal and tolerance in *C. elegans* was therefore investigated.

The gene *egl-3* encodes a *C. elegans* homolog of a mammalian proprotein convertase that participates in the processing of neuropeptide precursors in *C. elegans*. A mass spectrometry analysis showed that out of 75 neuropeptides normally detected in the

wild type N2, only one neuropeptide was detected in the mutant strain *egl-3 ok979*, which contains a 1578bp deletion in the *egl-3* gene (Husson et al., 2006). Thus the mutant is largely devoid of major classes of neuropeptides.

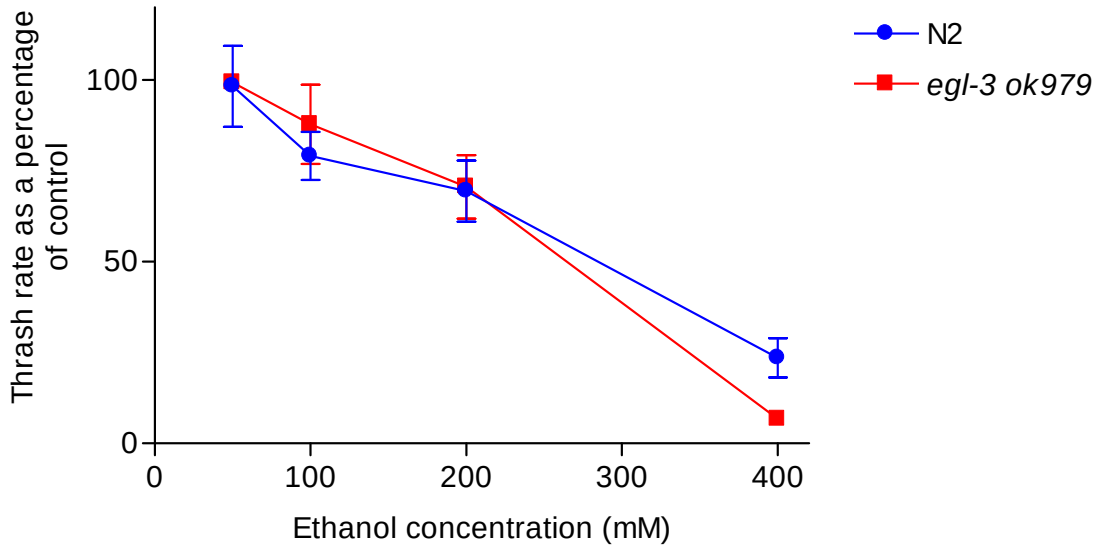


Figure 6.16 Rate of thrashes in ethanol as a percentage of basal rates of thrashes for N2 and *egl-3 ok979* worms. Results are the mean ±s.e. of ten independent worms. Mean rate of thrashes of worms in the absence of ethanol was 102.7/min for N2 and 84.95/min for *egl-3 ok979*. The basal rate of thrashing was thus significantly different (t77=4.299, P<0.0001).

The *egl-3 ok979* worms were used to determine if neuropeptides were involved in the effects of ethanol in *C. elegans*. First the response of *egl-3 ok979* worms to acute ethanol in the thrashing assay was investigated. N2 and *egl-3 ok979* worms behaved similarly over the concentration range 100-400mM (Figure 6.16). This makes it likely that neuropeptides are not involved in the acute effects of ethanol.

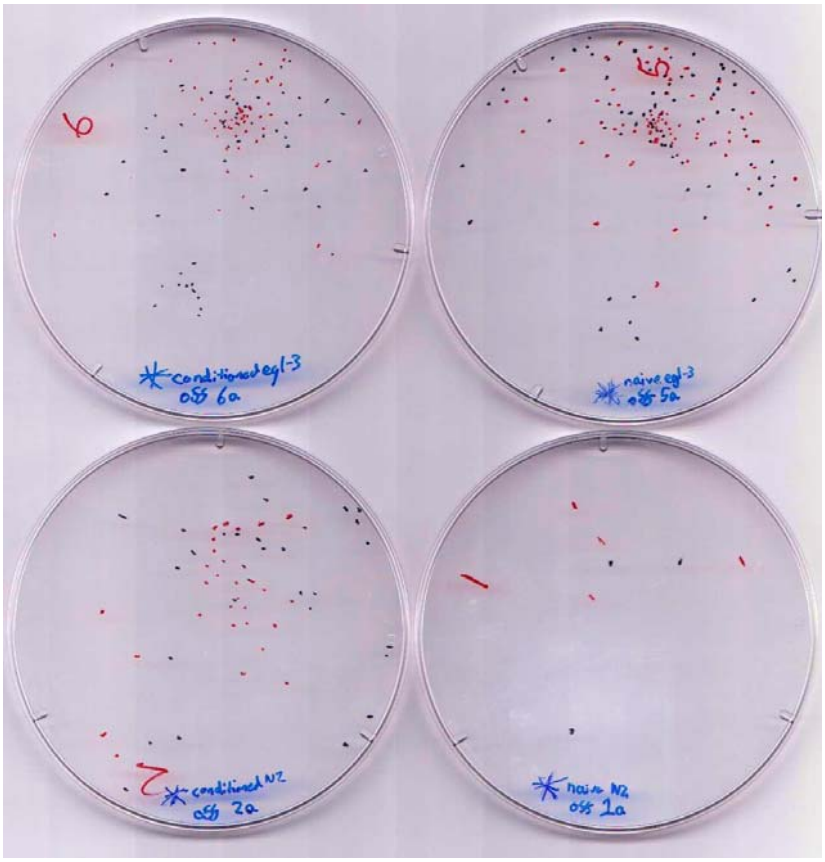


Figure 6.17 Photographs showing the positions of N2 (bottom) and *egl-3 ok979* (top) worms remaining on non-ethanol food race plates after two hours (red) and after three hours (black). Control worms are labelled 'naive' (right). Withdrawn worms have been exposed to 48 hours at 252mM ethanol and are labelled 'conditioned' (left). The blue stars show at which edge of the plate the spot of food that the worms were moving towards had been placed. In this experiment as worms reach the food they are removed hence the lower numbers of worms left on the naive N2 plate.

The response of *egl-3 ok979* mutants to ethanol conditioning was then investigated. First a food race was performed using these mutants under conditions of withdrawal, withdrawal relief or control conditions. However one of the phenotypes of loss of function mutations in *egl-3* is coiler behaviour which reduces the coordination of movement. Although this behaviour was not directly recorded in this assay, it is probably why, over a two hour period, less than 10% of the worms from any food race plate containing *egl-3 ok979* reached the food. The positions of the remaining worms relative to the food at the 2 hour and 3 hours time points were marked and the result was photographed (Figure 6.17). Overall this suggests that the food race assay is not

an appropriate assay to use to investigate the role of mutants that have locomotory impairment.

The spread of worms in the food races containing control and withdrawn *egl-3* worms appeared very similar, which might imply that there was no additive effect of withdrawal. If the withdrawal effect on locomotion was acting independently of the effect of the *egl-3* mutation on locomotion one might expect that the withdrawn *egl-3* worms would perform worse than the control *egl-3* worms. If this is not the case it suggests that they may act on the same pathway. The limitation of the locomotory phenotype with respect to defining drug induced effects which was discussed previously (see section 6.1), is well illustrated in this experiment.

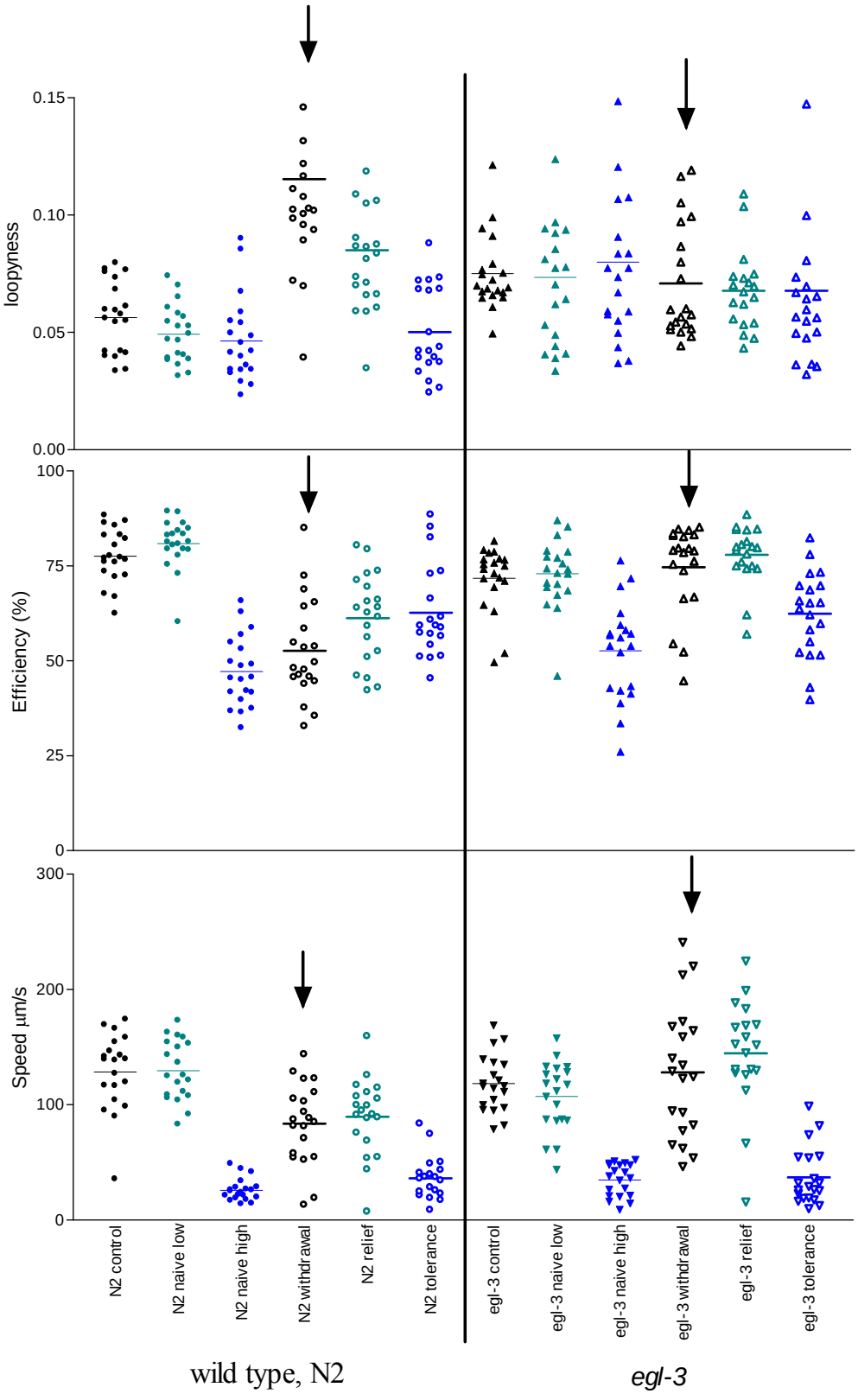


Figure 6.18 A mutant deficient in peptidergic signalling, *egl-3(ok979)* exhibits ethanol intoxication but not withdrawal. The data were collected and analysed as described in the legend to Figure 6.10. The data for wild-type controls are shown as circles and for *egl-3* as triangles. Each data point represents a measurement from a single worm and the bars indicate the mean for each data set. Arrows indicate data sets which will be discussed in the text. See Appendix B for statistical analysis.

Accordingly it was decided to extend the investigation of the effects of conditioning on *egl-3* mutants and several other candidate genes using the automated video analysis in order to overcome the confounds highlighted above. Videos were taken at the time point five minutes after worms were added to the food race.

The effect of intoxication, withdrawal, relief from withdrawal and tolerance on loopyness, efficiency and speed in *egl-3 ok979* worms and matched N2 worms was investigated by this method (Figure 6.18). Preconditioning and subsequent withdrawal induced expected changes in N2 in which the worms display reduced speed and efficiency and increased loopyness. No effect of withdrawal was detected in any of the parameters in the *egl-3* worms (see data sets marked by arrows in Figure 6.18) although they showed normal intoxication. The control *egl-3* worms appeared loopier than the control N2 worms indicating that they may partially phenocopy the effect of withdrawal.

It would therefore appear that the development of withdrawal behaviour in *C. elegans* requires the action of neuropeptides, but that they are unlikely to be involved in the acute effects of ethanol. It would therefore seem likely that they were involved in the process of neuroadaptation.

6.2.7 The role of GABA in the development of ethanol dependence

GABA receptors have been described as being among some of the major targets for ethanol in mammalian nervous systems (see Chapter 1). Additionally GABA is the major inhibitory neurotransmitter involved in normal *C. elegans* locomotion. ACh release on one side of the worm stimulates muscle contraction and also activates

contralateral GABAergic neurons, which leads to muscle relaxation on the opposite side of the worm. This enables the worm's body to bend producing sinusoidal locomotion. It has been demonstrated that the loopy behaviour seen in the withdrawn worms is not caused by increased ACh release (see section 6.2.5). This effect might be caused by decreased GABAergic signalling. It was therefore interesting to investigate to what extent GABAergic signalling was involved in intoxication and withdrawal in *C. elegans*.

To study this, worms with loss of function alleles of the genes *unc-25* and *unc-49* were used. The gene *unc-25* encodes the *C. elegans* ortholog of the GABA neurotransmitter biosynthetic enzyme glutamic acid decarboxylase which is required for GABA synthesis. The *unc-25 e156* worms that were used are thus deficient in GABA. The gene *unc-49* has multiple splice variants which each encode different subunits of a heteromeric $GABA_A$ receptor. The *unc-49 e407* allele that was used is a null mutation in one of the subunits of this receptor (UNC-49B), which is required to form functional $GABA_A$ receptors at the neuromuscular junction in body wall muscles (McIntire et al., 1993). Therefore the *unc-25* worms have a more general deficiency as they have a loss of function in all GABA signalling pathways, whereas the *unc-49* worms have a more specific loss of function in ionotropic GABAergic inhibition of the body wall muscle.

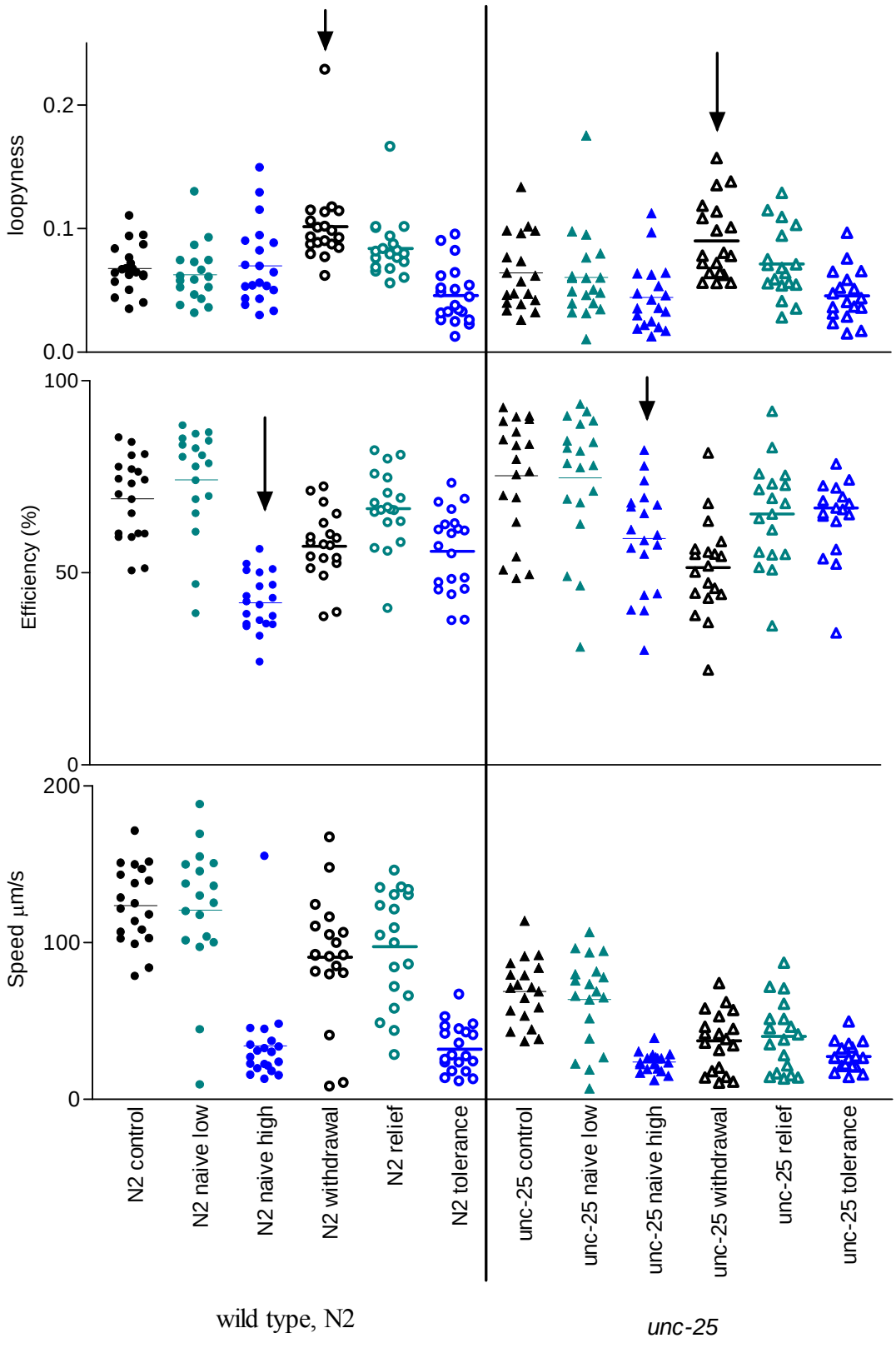


Figure 6.19 A mutant deficient in the neurotransmitter GABA, *unc-25 e156* exhibits subtle differences in intoxication and withdrawal. The data were collected and analysed as described in the legend to Figure 6.10. The data for wild-type controls are shown as circles and for *unc-25* as triangles. Each data point represents a measurement from a single worm and the bars indicate the mean for each data set. Arrows indicate data sets which will be discussed in the text. See Appendix B for statistical analysis.

The effect of ethanol conditioning on *unc-25* mutants was thus investigated first as this is the more general mutation. The effect of intoxication, withdrawal, relief from withdrawal and tolerance on loopyness, efficiency and speed in *unc-25 e156* worms was examined using the automated video analysis (Figure 6.19).

The N2 worms, as expected, showed increased loopyness and decreased efficiency and speed in the withdrawal condition, and decreased efficiency and speed in the intoxication condition. The main differences between the results for *unc-25 e156* worms and the N2 controls were that the *unc-25* worms showed a non-significant rather than significant increase of loopyness in the withdrawal condition and that they showed less of a decrease in efficiency in response to intoxication than N2 (both marked by arrows in Figure 6.19). In addition their speed was significantly lower than N2 in all conditions except intoxication and tolerance under which conditions the speed of the N2 worms was also very low.

These differences may indicate that there is a subtle effect of GABA signalling involved in intoxication and withdrawal in *C. elegans* in the absence of which both effects are slightly reduced. However it is clear that intoxication, withdrawal, relief from withdrawal and tolerance can all occur in worms severely deficient in the neurotransmitter GABA and thus GABAergic signalling is not likely to have a major role in the ethanol response.

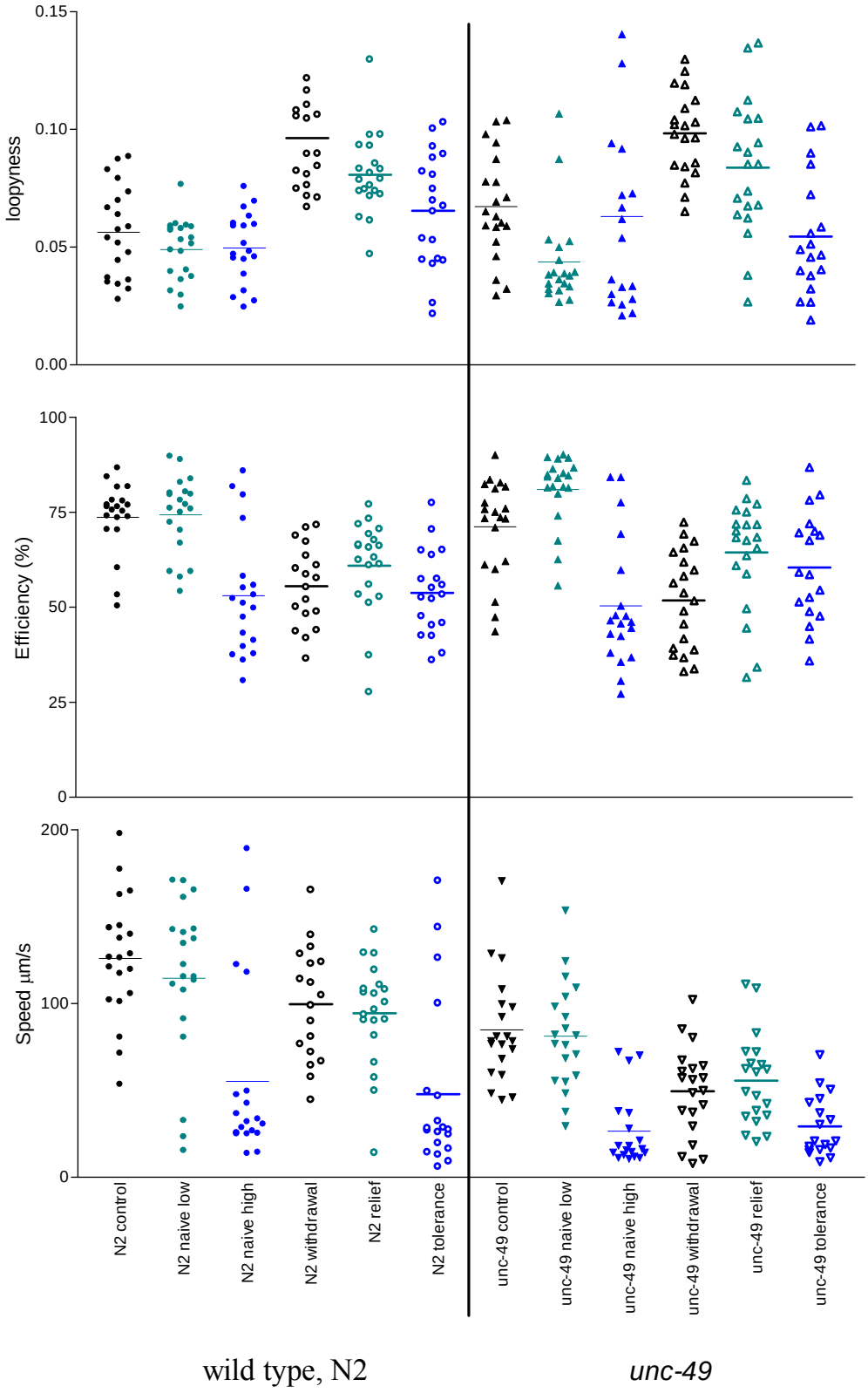


Figure 6.20 A mutant deficient in the neuromuscular junction $GABA_A$ receptor, *unc-49 e407* responds like wild-type N2 worms to acute and chronic ethanol. The data were collected and analysed as described in the legend to Figure 6.10. The data for wild-type controls are shown as circles and for *unc-49* as triangles. Each data point represents a measurement from a single worm and the bars indicate the mean for each data set. See Appendix B for statistical analysis.

In order to investigate if any possible effects of GABA signalling required the $GABA_A$ receptor the automated video analysis was used to investigate the effect of intoxication, withdrawal, relief from withdrawal and tolerance on loopyness, efficiency and speed in *unc-49 e407* worms (Figure 6.20). The *unc-49* worms moved more slowly than N2 in all conditions except tolerance. However they otherwise displayed a normal wild-type like response to ethanol conditioning, consisting of an increase in loopyness and decrease in efficiency and speed in response to withdrawal and a greater decrease in efficiency and speed in response to intoxication. This indicates that if GABA signalling is involved in the response to ethanol it is not acting through the $GABA_A$ receptor at the body wall muscle neuromuscular junction.

6.2.8 The response of a dopaminergic signalling mutant to ethanol conditioning

In mammalian systems the mesolimbic dopamine pathway which is involved in reward, is central to the development of dependence to all addictive drugs (see Chapter 1). In *C. elegans* dopaminergic signalling has been shown to be involved in regulating area restricted search and thus reversal frequency, which has been shown to be affected by ethanol conditioning (Hills et al., 2004).

The gene *cat-2* encodes tyrosine hydroxylase, an enzyme required for dopamine synthesis. The *cat-2 e1112* allele contains a nonsense mutation in *cat-2,* leading to depleted dopamine levels. The automated video analysis was used to investigate the effect of intoxication, withdrawal, relief from withdrawal and tolerance on loopyness, efficiency and speed in *cat-2 e1112* worms (Figure 6.21).

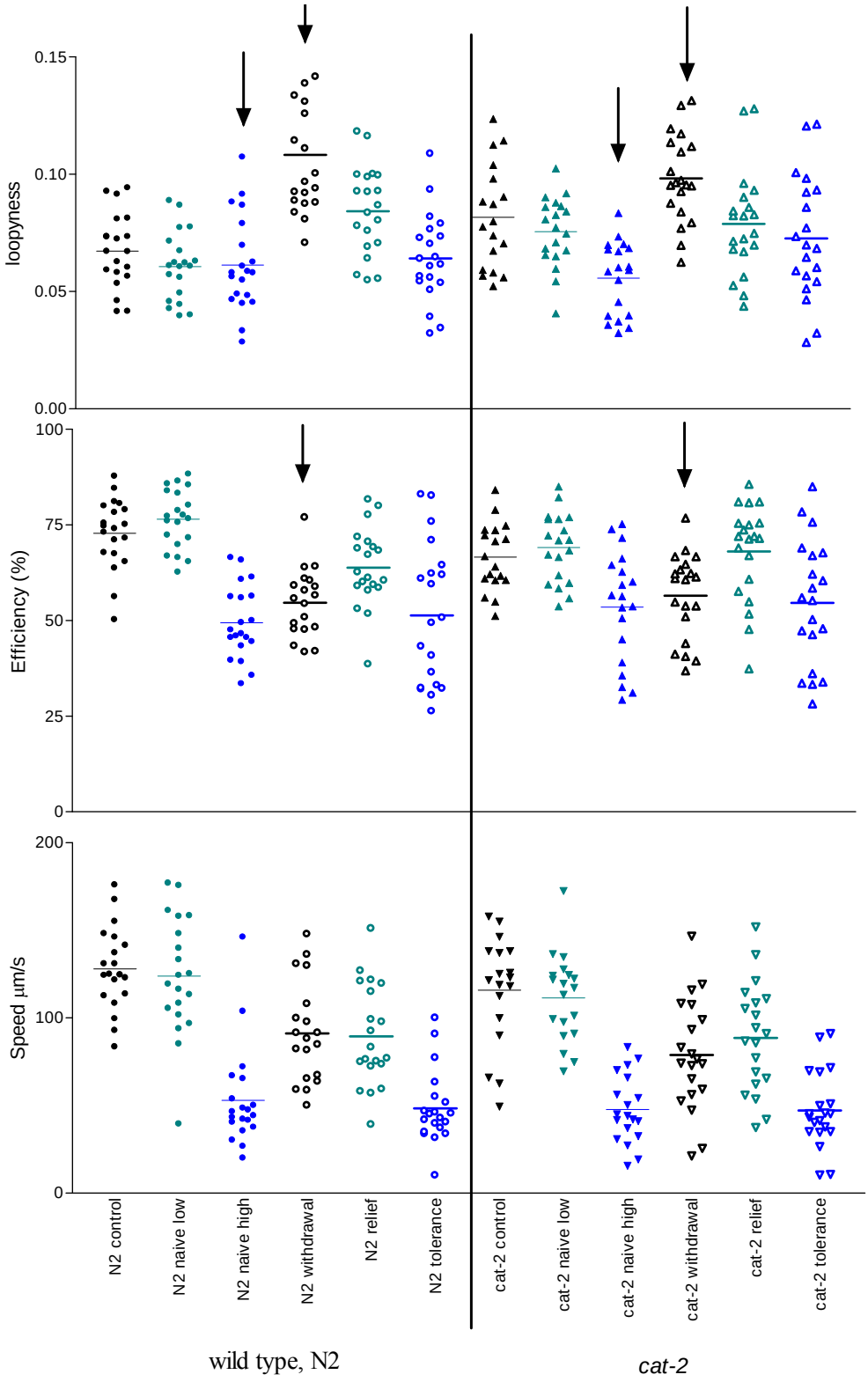


Figure 6.21 A mutant deficient in the neurotransmitter dopamine, *cat-2 e1112* exhibits subtle differences from N2 in intoxication and withdrawal. The data were collected and analysed as described in the legend to Figure 6.10. The data for wild-type controls are shown as circles and for *cat-2* as triangles. Each data point represents a measurement from a single worm and the bars indicate the mean for each data set. Arrows indicate data sets discussed in the text. See Appendix B for statistical analysis.

The N2 worms showed the expected increase in loopyness and decrease in efficiency and speed in response to withdrawal, and the expected greater decrease in efficiency and speed in response to intoxication. The main differences between the *cat-2* and the N2 worms were that the *cat-2* worms had a non-significant as opposed to significant increase in loopyness and decrease in efficiency in response to withdrawal. In addition they showed a significant decrease in loopyness in response to intoxication which was not seen in the N2 worms.

These changes are due to the fact that the control *cat-2* worms (and those acutely exposed to low dose ethanol) are significantly loopier and less efficient than the equivalent N2 worms. This could indicate that they are phenocopying the effect of withdrawal, which then does not have a fully additive effect. This would then imply that dopamine signalling could be involved in the response to ethanol in *C. elegans*. However it is clear that some of the pathways that lead to intoxication and withdrawal are still intact in the *cat-2* worms as both intoxication and withdrawal have their expected effect to decrease the speed of *cat-2* worms.

6.2.9 The role of glutamatergic signalling in the response to ethanol conditioning

Glutamatergic signalling, especially through the NMDA receptor, is implicated in the response to ethanol in mammalian systems (see Chapter 1).

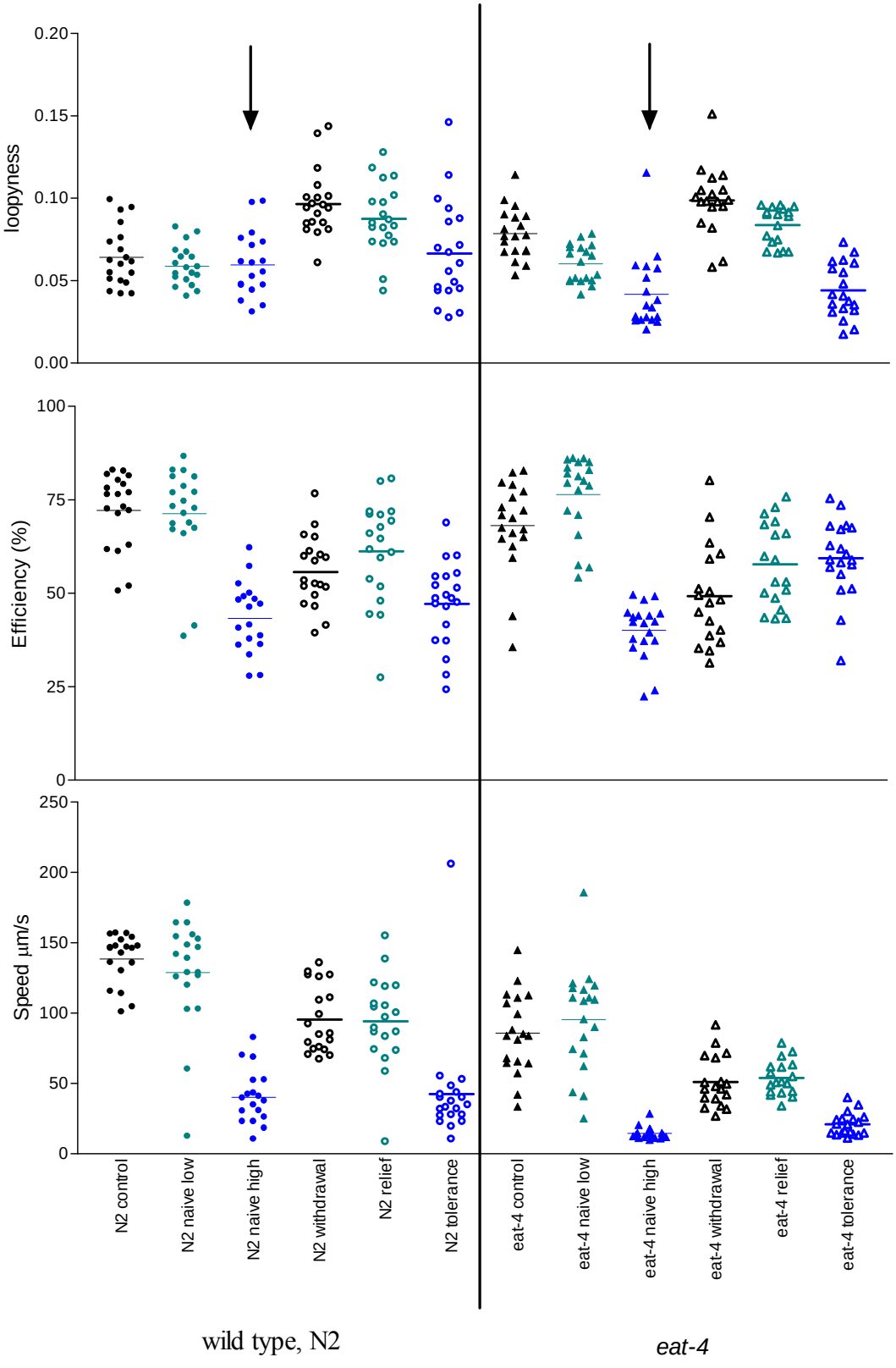


Figure 6.22 A mutant deficient in the neurotransmitter glutamate, *eat-4 ky5* exhibits subtle differences from N2 in response to intoxication. The data were collected and analysed as described in the legend to Figure 6.10. The data for wild-type controls are shown as circles and for *eat-4* as triangles. Each data point represents a measurement from a single worm and the bars indicate the mean for each data set. Arrows indicate data sets discussed in the text. See Appendix B for statistical analysis.

In *C. elegans* the gene *glr-2* which encodes a glutamate receptor was shown to be induced in response to ethanol exposure (Kwon et al., 2004). Additionally glutamatergic signalling in *C. elegans* has been shown to be involved in the regulation of reversal frequency, which has been shown to be affected by ethanol conditioning (Brockie et al., 2001;Hills et al., 2004). It was therefore interesting to investigate whether glutamatergic signalling was involved in the development of intoxication or withdrawal in *C. elegans*.

To do this, worms containing the *eat-4 ky5* allele were used. This is a loss of function allele of the gene *eat-4*. This gene encodes an ortholog of the mammalian BNPI vesicular glutamate transporter and loss of function in this gene results in severely reduced glutamate signalling (Lee et al., 1999). The automated video analysis was used to investigate the effect of intoxication, withdrawal, relief from withdrawal and tolerance on loopyness, efficiency and speed in *eat-4 ky5* worms (Figure 6.22).

The N2 worms, as in previous experiments, showed an increase in loopyness in response to withdrawal and a decrease in efficiency and speed in response to both intoxication and withdrawal, which was greatest under the intoxication condition. The main difference between the *eat-4 ky5* worms and the N2 worms was that the *eat-4* worms showed a significant decrease in loopyness in response to intoxication which the N2 did not. This may have been due to the control *eat-4* worms being significantly loopier than the control N2 worms. This may indicate that glutamate signalling has a slight, subtle role in the ethanol response, causing the *eat-4* worms to slightly phenocopy withdrawal. However there is still a clear effect of withdrawal in the *eat-4*

worms indicating that these pathways are relatively unaffected by the loss of all glutamatergic signalling.

One additional difference is that the *eat-4* worms had a reduced speed compared to N2 under all conditions.

6.2.10 The role of 5-HT signalling in the response to ethanol conditioning

5-HT signalling has been implicated in the development of ethanol dependence in mammalian systems (see Chapter 1). To investigate its role in intoxication and withdrawal in *C. elegans,* worms with a loss of function mutation in the gene *tph-1* were used. This gene encodes tryptophan hydroxylase, the enzyme that encodes the rate limiting step in 5-HT biosynthesis. It is required for 5-HT biosynthesis *in vivo*.

The automated video analysis was used to investigate the effect of intoxication, withdrawal, relief from withdrawal and tolerance on loopyness, efficiency and speed in *tph-1 mg280* worms (Figure 6.23).

The N2 worms showed the expected increase in loopyness in response to withdrawal and a decrease in efficiency and speed in response to both intoxication and withdrawal, which was greatest under the intoxication condition. The main differences between the *tph-1* worms and the N2 worms were that the *tph-1* worms didn't show a significant effect of withdrawal on loopyness or efficiency of movement, and also didn't show a significant effect of intoxication on efficiency. The

tph-1 worms did however show a significant effect of both intoxication and withdrawal on speed of movement.

Compared to the N2 control worms the *tph-1* control worms showed increased loopyness, increased variability in loopyness and decreased efficiency and speed of movement. The *tph-1* worms may thus be partially phenocopying the withdrawal response.

This indicates that it is likely that 5-HT signalling has a role in the response to ethanol in *C. elegans*. However it is clear that there are other pathways involved as an effect of both intoxication and withdrawal is still detectable in the absence of 5-HT signalling.

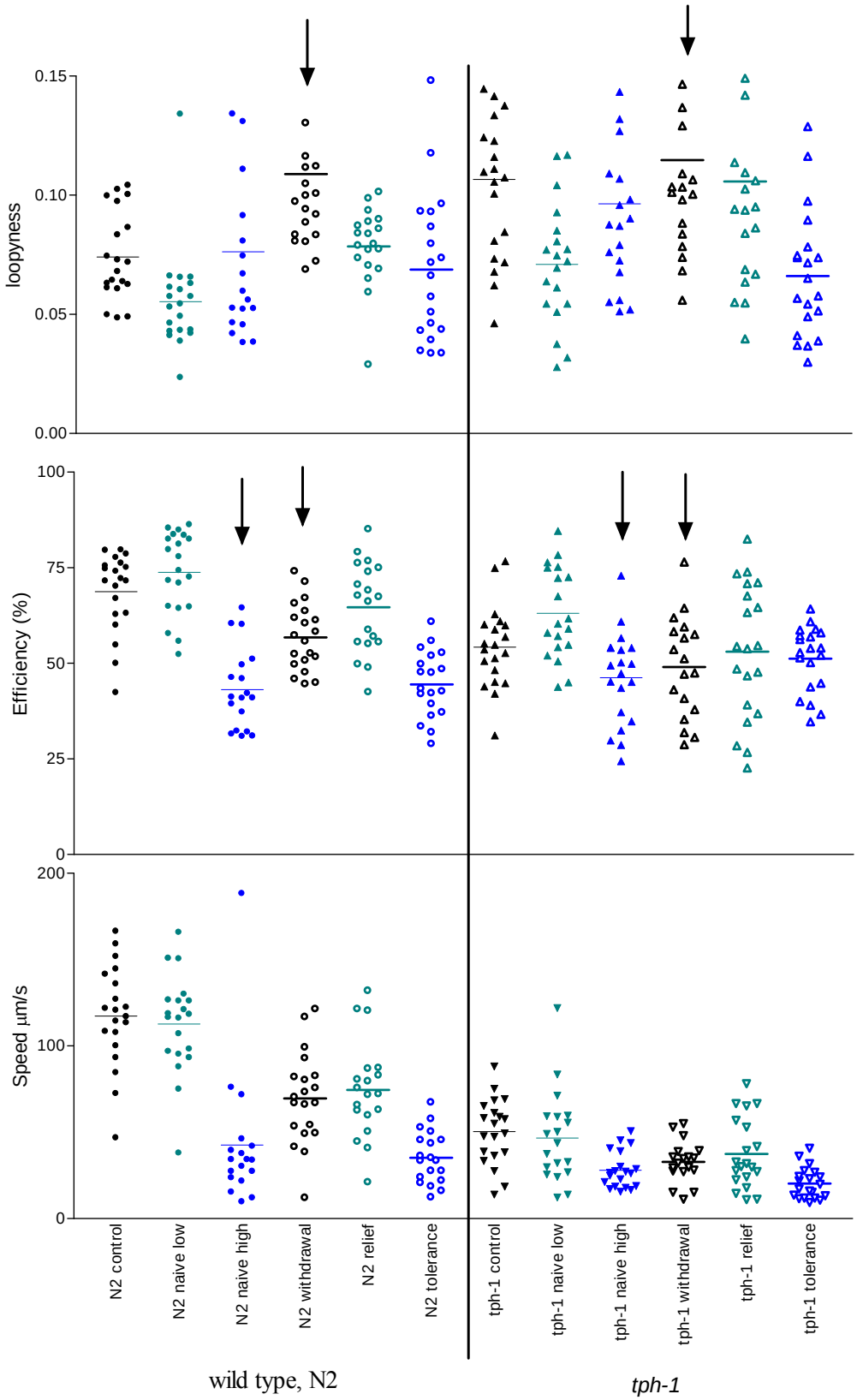


Figure 6.23 A mutant deficient in the neurotransmitter 5-HT, *tph-1 mg280* shows a reduced effect of both intoxication and withdrawal. The data were collected and analysed as described in the legend to Figure 6.10. The data for wild-type controls are shown as circles and for *tph-1* as triangles. Each data point represents a measurement from a single worm and the bars indicate the mean for each data set. Arrows indicate data sets discussed in the text. See Appendix B for statistical analysis.

6.2.11 Summary of N2 video analysis results

Including the original N2 data shown in Chapter 5, a total of eight sets of video analysis data for the N2 controls were collected. These were used to understand the variability in the wild-type video analysis data so that important differences between the N2 data and the mutant data could be focussed on and criteria set for the significance of the results.

Percentage of eight N2 data sets in which a significant difference (P<0.05) was present between stated conditions			
	Loopyness	**Efficiency**	**Speed**
Control – Withdrawal	100.0	100.0	87.5
Naive low (low dose ethanol on naive worms) – Relief from withdrawal (low dose ethanol on conditioned worms)	62.5	37.5	50.0
Withdrawal – Relief from withdrawal	37.5	12.5	0.0
Control – Intoxication	0.0	100.0	100.0
Control – Tolerance	0.0	100.0	100.0
Intoxication - Tolerance	12.5	37.5	0.0

Table 6.2 Percentage of eight N2 data sets of approx 20 worms per condition in which a significant difference was present between the listed conditions.

Using this analysis it is clear that 100% of the N2 data sets have a significant difference between control and withdrawal in the loopyness and efficiency parameters and between control and intoxication in the efficiency and speed parameters. Therefore a lack of a significant difference between these conditions in data sets from mutant strains will be considered to be an important difference. It is also clear that in the video analysis the presence of a significant effect of relief from withdrawal or tolerance is variable. Data sets from mutant strains which lack an effect of relief from withdrawal or tolerance thus cannot be considered to prove that these effects are not occurring in these mutant strains. This is less consistent than in the food race where only 3 out of 22 experiments fail to show a relief from withdrawal effect.

A summary of the major differences based on these criteria:

A summary of mutant strains which have major differences from wild type						
	Withdrawal			Intoxication		
	Loopyness	Efficiency	Speed	Loopyness	Efficiency	Speed
slo-1						
egl-3	n	n	n			
unc-25	n					
unc-49						
cat-2	n	n		y		
eat-4				y		
tph-1	n	n			n	

Table 6.3 A summary of mutant strains with major difference from wild type. n = Absence of an expected significant difference. y = Presence of an unexpected significant difference.

This shows that the *egl-3* worms show no effect of withdrawal whereas all the other mutant strains show at least some effect of both withdrawal and intoxication. This indicates that neuropeptide signalling is required for the development of withdrawal. The *cat-2* and *tph-1* mutant strains show major differences from wild type in three out of six comparisons. This makes it possible that dopamine and 5-HT signalling have some role in the ethanol induced response. This will be considered further in the discussion (section 6.3).

6.3 Discussion

This chapter has investigated the involvement of eight candidate signalling pathways in the development of neuroadaptation to ethanol in *C. elegans*. In summary the results have shown that neuropeptide signalling is required for ethanol withdrawal and that 5-HT and dopamine signalling may also be involved in the ethanol response. They do not however demonstrate a major role for the BK potassium channel or the neuropeptide receptor NPR-1 in ethanol intoxication, tolerance or withdrawal, thus largely contradicting the interpretation made from previous observations (Davies et al., 2003;Davies et al., 2004a). The signalling pathways examined will now be discussed in turn.

6.3.1 Neuromodulatory transmitters are involved in the adaptive response to ethanol

Neuropeptides

This study has shown that worms containing the *egl-3 ok979* allele do not show withdrawal behaviour following six hours exposure to ethanol (Figure 6.18). They do, however, show normal intoxication in response to acute ethanol (Figures 6.16 and 6.18). The *egl-3* gene encodes a *C. elegans* homolog of a mammalian proprotein convertase that participates in the processing of neuropeptide precursors in *C. elegans.* Thus the worms containing the null *egl-3 ok979* allele are almost totally lacking in neuropeptides (Husson et al., 2006). Therefore it must be concluded that neuropeptides are required for the development of the locomotory behaviour of withdrawal in *C. elegans*.

However the *egl-3* mutant does show a small effect of tolerance on efficiency of locomotion. There is a significant difference between the control *egl-3* worms and the intoxicated *egl-3* worms, whereas the difference between the control and tolerant *egl-3* worms was not significant. This may indicate that the processes by which withdrawal and tolerance develop are distinct.

There are at least 28 FMRFamide-like peptide genes (*flp*), 42 neuropeptide-like protein genes (*nlp*) and 38 insulin-like peptide genes in *C. elegans* (Husson et al., 2006). The only study that has investigated the interaction between ethanol and neuropeptides in *C. elegans* is the work done on the neuropeptide Y receptor-like protein NPR-1, which has been previously described (Davies et al., 2004a). Withdrawal behaviour in the food race is still present in worms which lack this neuropeptide receptor; therefore other peptides and peptide receptors must be responsible for the development of withdrawal.

Neuropeptides in general act through G-protein coupled metabotropic receptors to produce long term modulatory responses (Li and Kim, 2008). They therefore probably cause the development of withdrawal through these neuromodulatory methods. Neuropeptide release is unlikely to be a direct target of ethanol as lack of neuropeptide signalling does not affect intoxication, but neuropeptides may be released further downstream from the ethanol target and cause homeostatic alterations in signalling in response to chronic ethanol exposure. In mammalian systems many peptides are known to be involved in the development of alcohol dependence (see Introduction section 1.5 and 1.7). These include the opioid peptides, neuropeptide Y (NPY) and corticotrophin releasing factor (CRF) (Heilig et al., 1994;Herz, 1997).

These do not have direct peptide homologs in *C. elegans*, although the *C. elegans* genome does encode NPY receptor-like neuropeptide receptors (Li and Kim, 2008).

Serotonergic signalling

5-HT signalling has been implicated in the development of ethanol dependence in mammalian systems (see Chapter 1). To investigate its role in intoxication and withdrawal in *C. elegans, tph-1 mg280* worms which are deficient in 5-HT were used.

The *tph-1 mg280* worms had a reduced response to both intoxication and withdrawal in the loopyness and efficiency parameters but still displayed a response to intoxication and withdrawal when speed was measured (Figure 6.23).

It therefore seems likely that serotonergic signalling is involved in both intoxication and withdrawal, as in the absence of 5-HT, parts of both of these behaviours are reduced. The *tph-1* mutants, which lack 5-HT, behave in a similar manner to the withdrawn worms. In mammalian systems ethanol elevates levels of 5-HT in various areas of the extended amygdala and forebrain (Daws et al., 2006;McBride et al., 1993). In addition excitation of the 5-HT_3 receptor is implicated as one of the major targets for ethanol (Campbell and McBride, 1995). It is possible from the data that an increase in 5-HT signalling could be involved in intoxication and a decrease of 5-HT signalling in withdrawal.

However as both intoxication and withdrawal do still occur in the absence of 5-HT signalling other pathways are clearly also involved in these responses.

Dopaminergic signalling

In mammalian systems the mesolimbic dopamine pathway which is involved in reward, is central to the development of dependence to all addictive drugs (see Chapter 1) In *C. elegans* dopaminergic signalling has been shown to be involved in regulating area restricted search and thus reversal frequency, which this study has shown to be affected by ethanol conditioning (Hills et al., 2004).

The behaviour of *cat-2 e1112* worms which lack an enzyme involved in dopamine synthesis was investigated in the automated video analysis (Figure 6.21). In the control conditions the *cat-2* worms' locomotion was significantly loopier and less efficient than the controls. This pattern was not found in the intoxicated, tolerant, withdrawn or relief worms. This resulted in a non-significant rather than significant increase in loopyness and decrease in efficiency in response to withdrawal in *cat-2* mutants and in a significant decrease in loopyness in the intoxicated *cat-2* mutants

It is possible that this indicates that there is no additive effect of *cat-2* and withdrawal. This may mean that some of the effects that withdrawal has on loopyness and efficiency occur downstream of decreased dopamine release but in the same pathway. It is unlikely to indicate that ethanol directly affects dopamine release as in that case one would expect to see a reduced effect of intoxication, as well as a reduced effect of withdrawal.

Further investigation of the dopaminergic pathway may therefore be interesting. For example dopamine signalling is involved in the slowing response when worms move onto food. Worms with loss of function mutations in *cat-2* do not display a slowing

response on food (Sawin et al., 2000). It would be interesting to see if withdrawn worms showed an altered slowing response.

Interim Summary

Thus neuromodulatory transmitters such as neuropeptides, 5-HT and dopamine have been shown in these experiments to have the greatest effect on the development of neuroadaptation to ethanol in *C. elegans*. It is possible to speculate that initial ethanol intoxication might lead to the release of neuropeptides which act in some manner to decrease 5-HT and dopamine release leading to withdrawal. However further research will be needed to confirm a role for 5-HT and dopamine in this response. It is interesting to note that a recent paper implicated *cat-2* and *tph-1* mutants in the development of preference for ethanol after ethanol conditioning (Lee et al., 2009). These mutants are also involved in behavioural plasticity in response to food and starvation in mammals and worms (Sawin et al., 2000).

6.3.2 Classical fast transmitters do not appear to be involved in the adaptive response to ethanol

Acetylcholine release

Increased acetylcholine release has been shown to cause loopy body bends behaviour (McMullan et al., 2006) as does ethanol withdrawal. However neither ethanol intoxication (Figure 6.14) nor withdrawal (Figure 6.15) affected sensitivity to aldicarb in *C. elegans*. This means that neither affects acetylcholine release at the *C. elegans* neuromuscular junction.

This is interesting as many proteins that are involved in neurotransmitter release have been shown to be involved in the acute effects of ethanol. For example a null allele of the gene *rab-3*, which encodes a small G-protein which interacts with synaptic vesicles to regulate their release, confers ethanol resistance (Kapfhamer et al., 2008). The RAB-3 protein has been implicated in the release of small clear vesicles containing neurotransmitter rather than large dense core vesicles containing neuropeptides, however it is possible that it also has a role in neuropeptide release (Xu and Xu, 2008). These mutants are also aldicarb resistant, indicating that they have reduced ACh release. In addition a single nucleotide polymorphism D214N in the gene *unc-18*, which encodes a syntaxin binding protein, causes slower individual fusion events and has been shown to confer ethanol resistance (Graham et al., 2008), although worms carrying this mutation show normal aldicarb sensitivity. As previously mentioned *slo-1* loss of function mutants have been described as being resistant to ethanol although only a slight effect of this has been shown in this study (Davies et al., 2003). This gene encodes a BK potassium channel and loss of function mutations in this gene have been shown to increase quantal content at the neuromuscular junction primarily by increasing the duration of release (Wang et al., 2001). These mutants are hypersensitive to aldicarb

So mutations that cause resistance to ethanol do not consistently reduce or increase ACh release although many are involved in neurotransmitter release in some capacity. Neither ethanol intoxication nor withdrawal affects ACh release. It therefore seems likely that these genes are affecting the ethanol response in a location other than the neuromuscular junction.

GABAergic signalling

In mammalian systems the $GABA_A$ receptor has been described as one of the major targets for ethanol (see Chapter 1) and there have also been some studies linking the $GABA_B$ receptor to the ethanol response (Dzitoyeva et al., 2003;Littleton and Little, 1994). The strains *unc-25 e156*, which is deficient in GABA, and the more specific *unc-49 e407*, which lacks the $GABA_A$ receptor found at the neuromuscular junction were used, in order to investigate the role of GABAergic signalling in intoxication and withdrawal in the worm.

Our studies show very slight alteration in the response of the *unc-25* worms to ethanol withdrawal which is not found in the *unc-49* worms (Figures 6.19 and 6.20). This indicates that if GABAergic signalling is involved in the locomotory response to ethanol it is not acting through the $GABA_A$ receptor at the neuromuscular junction in body wall muscles.

There are other GABA receptors encoded by the genome. One of these, EXP-1 controls defecation and so is unlikely to be the cause of alterations in locomotion. However there are three other potential $GABA_A$ receptor subunits encoded by the genome, which have not yet been characterized (Jorgensen, 2005). A $GABA_B$ receptor has also recently been described in *C. elegans* (Dittman and Kaplan, 2008). It is likely that one or more of these controls the foraging movements of the head, which are affected by GABA release from the RME neurons, and which could affect the measurement of loopyness. These pathways could therefore be involved in a small part of the ethanol response.

Glutamatergic signalling

To investigate the role of glutamatergic signalling in intoxication and withdrawal in *C. elegans* worms containing the *eat-4 ky5* allele which is a loss of function allele of the gene *eat-4* were used. This gene encodes an ortholog of the mammalian BNPI vesicular glutamate transporter and loss of function in this gene results in severely reduced glutamate signalling (Lee et al., 1999).

The only notable different in ethanol-related behaviour between *eat-4* worms and N2 was that both the intoxicated and tolerant worms were significantly less loopy than their wild type counterparts (Figure 6.22). This led to a significant difference in loopyness between control *eat-4* mutants and intoxicated *eat-4* mutants, which did not occur in any of the N2 controls. This could indicate that glutamatergic signalling plays a slight, inhibitory role in the acute ethanol response, but not in the development of neuroadaptations leading to withdrawal.

C. elegans contains many genes encoding glutamate receptor subunits including ionotropic receptors with similarity to AMPA and kainite receptors (*glr-1-8*), NMDA-like receptors (*nmr-1-2*), a group of glutamate gated chloride channels (*glc-1-4* and *avr-14-15*) and metabotropic glutamate receptors (*mgl-1-3*) (Brockie and Maricq, 2006). It has been shown that the gene *glr-2* is upregulated in *C. elegans* after 15 minutes exposure to ethanol and remains upregulated even after 6 hours ethanol exposure (Kwon et al., 2004). This could be a response to an involvement of glutamatergic signalling in the acute response to ethanol.

In mammalian systems inhibition of the NMDA receptor has been identified as one of the major targets for ethanol (Krystal et al., 2003), although studies have also shown roles for AMPA, kainate and metabotropic glutamate receptors (Sanchis-Segura et al., 2006;Carta et al., 2006). Further investigation is needed to see which receptors are involved in the decreased loopyness in response to intoxication in *C. elegans*. It is interesting that a reduction of glutamate levels leads to an increase rather than a decrease in ethanol induced behaviour in *C. elegans*. This would be consistent with ethanol and glutamate having antagonistic effects.

Interim Summary

Thus the classical fast transmitters GABA, ACh and glutamate have been shown to have limited roles, if any, in the effects intoxication and withdrawal on locomotion on food race plates in *C. elegans*. This is surprising as, in mammalian systems, $GABA_A$ and NMDA receptors have been strongly implicated in the acute response to ethanol. It is possible that either different protein sequences or different membrane compositions in *C. elegans* mean that ethanol acts on subtly different target proteins. Investigation of which targets it is acting on in *C. elegans* could further understanding of how ethanol interacts with its target proteins.

6.3.3 Genes previously implicated in the ethanol response in *C. elegans*.

The BK potassium channel, SLO-1

As previously described null mutations in the gene *slo-1* have been reported to produce phenotypes of at least partial ethanol resistance (Davies et al., 2003). This resistance to ethanol's acute effects might be expected to reduce the appearance of

tolerance (Pietrzykowski et al., 2004). In mammalian systems BK channels have been shown to be potentiated by ethanol and to reduce this potentiation over a long exposure to ethanol causing tolerance to its effects (Pietrzykowski et al., 2004). If the channels are not there to be activated, their activation will not reduce either. However there are likely to be other factors involved in both the acute response to ethanol and the development of tolerance.

Our results did not show this resistance to the inhibition of movement by ethanol in either the thrashing assay (Figures 6.4 and 6.5), the body bends assay (Figure 6.6) or the food race assay (Figure 6.7). These are all assays for the acute effect of ethanol and they show that *slo-1 js379* worms are as sensitive to ethanol as the wild type N2.

In order to ascertain that this effect was not due to the strain used worms containing the loss of function *slo-1* alleles *slo-1 pd23* and *slo-1 pd24* were also tested for ethanol resistance. Both strains showed responses to acute ethanol that were similar to the wild type controls in the thrashing assay.

So what could be the explanation for the discrepancy between the results and the published observations? One explanation could be that different assays were used. In this context it is interesting to note that the *slo-1 js379* worms move significantly faster than N2 on the food race plates in the naive high (intoxicated) condition according to the automated analysis (Figure 6.11), which is consistent with the description by Davies et al. of the *slo-1 js379* worms showing less of a reduction of speed on plates in the presence of ethanol (Davies et al., 2003). It is possible that this difference in speed on plates does not involve a difference in rate of body bends or a

difference in ability to reach the food in the food race and therefore was not detected in these assays.

However the *slo-1* mutants were picked out of a screen which involved them moving towards either a ring of food or a point source of butanone whilst acutely exposed to ethanol (Davies and McIntire, 2004). This is very similar to the food race assays, in which the worm moves towards a point source of food, and in which this study did not show a difference in the ability of *slo-1* mutants to move towards the food source. It is possible that the increased speed compared to N2 of the *slo-1* mutants on ethanol enables them to reach the ring of food more quickly, but that other aspects of the ethanol response prevent them navigating towards a point source of food. It is therefore possible that the ethanol resistance of *slo-1* mutants is a more subtle and specific effect on speed on plates which does not affect the rate of thrashes or body bends or their ability to navigate towards a point source of food.

The response of *slo-1* mutants to the ethanol conditioning assays was then investigated. The *slo-1* mutants show clear intoxication and withdrawal responses in both the food race (Figure 6.8) and the automated video analysis (Figure 6.10). However in the food race chronic conditioning with ethanol does not produce tolerance to intoxication and a low dose of ethanol does not relieve the withdrawal effect. This means that this experiment does not categorically demonstrate that *slo-1* mutants show neuroadaptation in response to chronic ethanol conditioning despite an apparent withdrawal effect. However using the automated video analysis *slo-1 js379* worms show a significant effect of relief from withdrawal on the efficiency and loopyness of their locomotion. This is a demonstration that *slo-1* mutants can develop

neuroadaptation in response to chronic ethanol conditioning. They do not show an effect of tolerance in this assay, but the N2 control worms also vary in whether they show an effect of tolerance in the video analysis assay (see section 6.2.11).

So, in conclusion *slo-1* loss of function mutants have a more subtle resistance to ethanol intoxication than previously described and they can develop neuroadaptation in response to chronic ethanol conditioning. However this study cannot state definitively whether or not the *slo-1* mutants develop tolerance to ethanol, despite no tolerance being detected in *slo-1* mutants, as the N2 results were also variable.

The NPY receptor like protein NPR-1

Worms with mutations in *npr-1* have been shown to show greater tolerance to ethanol and the *npr-1* gene has also been implicated in withdrawal (Davies et al., 2004a). From these findings it was suggested that acute ethanol could activate the NPR-1 pathway leading to its downregulation during chronic ethanol exposure. This would explain why mutants with lower function alleles of *npr-1* gain tolerance to ethanol's effects faster than N2 and show a phenotype similar to ethanol withdrawal which can be alleviated by acute ethanol (Davies et al., 2004a).

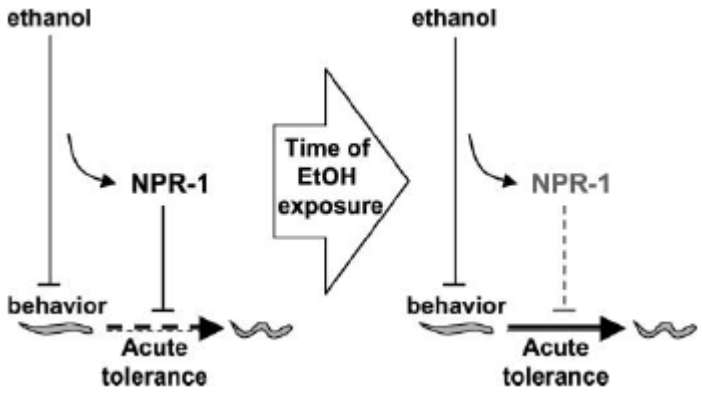


Figure 6.24 Diagram from (Davies et al., 2004a) illustrating the proposed role of NPR-1 in the development of acute tolerance. In this ethanol would activate the NPR-1 pathway acutely, but this would cause its downregulation over time, leading to tolerance to the acute effect of ethanol.

However both N2 worms and the Hawaiian strain CB4856 which has a lower function allele of *npr-1* were exposed to ethanol in the thrashing assay for a three hour continuous period and no acute tolerance development was seen in either (Figure 6.12). This is the same strain as was used in the published experiments and the experiments were conducted at the same ethanol concentrations. There are two possible explanations. One of these is that worms respond differently to ethanol in the thrashing assay than they do in an assay measuring speed on plates. This could be due to the pathways that lead to this form of movement not being affected by NPR-1. In this context it has been recently shown that thrashing and crawling on plates are distinct forms of locomotion distinguished by distinct kinematics and different underlying patterns of neuromuscular activity (Pierce-Shimomura et al., 2008). Another possibility is that the results in the published paper were affected by ethanol evaporation. This latter possibility was discounted in the paper by measuring internal ethanol concentration of the worms using the method that was shown earlier in this study to not perform this function. However the results for the CB4856 worms are probably too distinct to be caused by evaporation so the first possibility seems more likely.

The behaviour of worms containing the *npr-1* null allele *ky13* to ethanol conditioning was also investigated. This has a null mutation in the *npr-1* gene, whereas the Hawaiian strain that was used for the other assay has a lower function allele of the *npr-1* gene. The *npr-1 ky13* allele should therefore produce a similar but more pronounced phenotype.

It had been shown that after 18-22 hours exposure to 350mM ethanol, N2 animals when withdrawn from the ethanol show a tendency to display clumping and bordering activity (Davies et al., 2004a). This is when animals aggregate on the edges of the bacterial lawn, where the bacteria are thickest, rather than spreading all over the lawn and feeding in a solitary manner. This is a phenotype of *npr-1* null or lower function mutations such as *ky13* or CB4856. Davies et al. also showed that when *npr-1 ky13* worms were added to acute ethanol, their clumping behaviour was suppressed. This led to the hypothesis mentioned above, that ethanol activated the NPR-1 pathway, causing a consequent downregulation of the pathway over time which was revealed when ethanol was removed (see Figure 6.24).

The conditioned food race assay shows that naive *ky13* worms are not impaired in their ability to reach the food in the food race in the absence of ethanol. This assay also showed that *ky13* is not affected differently to N2 by ethanol withdrawal or acute ethanol, but it is faster in the food race assay under all of the conditions investigated.

Thus the withdrawal effect seen is not due to worms aggregating at the start point. This was unlikely anyway as aggregation is a phenotype that is seen on food on the thickest part of the bacterial lawn and in the assay the worms are away from food and have been washed to remove all bacteria from them. This also shows that NPR-1 is unlikely to be involved in the withdrawal behaviour that has been demonstrated in the food race assay.

It has previously been mentioned that chronic ethanol exposure may lead to long term behavioural consequences that persist once ethanol has been removed and yet are not

due to neuroadaptation. One of the causes of these consequences could be food deprivation as feeding rates are reduced by acute ethanol (Mitchell et al., 2007). Social feeding behaviour such as clumping is increased in wild type worms in response to food deprivation (de Bono and Bargmann, 1998). It is therefore possible that the clumping behaviour previously reported as a withdrawal behaviour was, in fact, a response to food deprivation by the chronically ethanol exposed worms. However this does not explain the decrease in clumping behaviour seen in *npr-1* worms in response to acute ethanol (Davies et al., 2004a). It is therefore likely that multiple pathways are involved in development of ethanol withdrawal, one of which is NPR-1 dependent and affects social feeding and one of which is NPR-1 independent and affects the food race.

6.3.4 Summary

The aim of this chapter was to investigate how the neuroadaptation to ethanol occurs, by investigating which genes, and therefore proteins, are required. This study has shown that the development of withdrawal behaviour requires neuropeptide signalling, although this is not involved in the acute response to ethanol. It has also shown that both 5-HT and dopamine signalling are likely to be involved in both intoxication and withdrawal.

The results show less of a clear effect of the classical fast transmitters GABA, glutamate and ACh on intoxication or withdrawal. However they could indicate a possible subtle role for GABAergic signalling in neuroadaptation to ethanol, although not through the UNC-49 body wall $GABA_A$ receptor. There is potentially also a role for glutamatergic signalling in acute intoxication. These roles require further investigation to be confirmed.

A slight effect of the BK channel on speed during intoxication was detected, but in the assays shown here this does not affect ability to reach food in the food race, or the rate of body bends or thrashes. No other clear effects of the BK channel on the ethanol response were seen. Additionally no effect of mutations in *npr-1* on tolerance in the thrashing assay or withdrawal in the food race assay were detected. This does not mean that NPR-1 is unaffected by ethanol, only that it is not involved in the effects of ethanol that seen in the food race.

Chapter 7 - Discussion

7.1 Principle findings

The aim of this thesis was to develop and utilise *C. elegans* as a model for alcohol dependence by investigating its response to acute and chronic ethanol exposure in wild type and mutant genetic backgrounds.

The main findings were:

- Ethanol is likely to equilibrate rapidly across the worm cuticle; therefore the internal concentration can be predicted from the external concentration in which the animal is placed.
- *C. elegans* exhibit the distinct and opposing ethanol-induced behavioural states of intoxication and withdrawal.
- *C. elegans* exhibits the phenomena of withdrawal relief, supporting the contention that the withdrawal effect observed is a result of neuroadaptation.
- Peptidergic signalling is key to the chronic adaption to, but not to the acute effects of, ethanol.
- Serotonergic and dopaminergic signalling may also be involved in the ethanol response in *C. elegans*.

This chapter addresses some of the broader implications of this work such as why the internal ethanol concentration is important, how neuroadaptation to ethanol can be distinguished from other chronic effects of ethanol and how *C. elegans* can be used as a model for alcohol dependence. The potential roles of the candidate molecules identified as part of the development of alcohol dependence in *C. elegans* are discussed, and how future work may be directed at establishing the mechanism for

this process is considered. Finally it is considered how this study relates to the study of alcohol dependence in humans.

7.2 The internal ethanol concentration.

The results described in Chapter 4 indicate that the *C. elegans* cuticle does not seem to be a significant diffusion barrier for ethanol when measuring behavioural consequences of ethanol exposure. As discussed in Chapter 4, under these circumstances it is likely that the internal ethanol concentration of the worm is similar to the bath solution.

Thus, whilst *C. elegans* and humans have a qualitatively similar response to ethanol consisting of possible hyperactivity at low doses, followed by sedation at higher doses and anaesthesia and eventual death at even higher doses, the exact doses involved are different. *C. elegans* display subtle intoxicating effects at 10-100mM and more sedative effects at 100-300mM, as opposed to intoxicating doses of 10-40mM and sedative ones of 40-90mM in humans. Above 300mM the increasing reduction in the ability of *C. elegans* to perform normal rhythmic behaviours could be considered similar to a human undergoing respiratory depression in response to alcohol poisoning (Lamminpaa and Vilska, 1990).

The comparative resistance to alcohol displayed by *C. elegans* could indicate that they may have evolved in environments where higher levels of ethanol were frequently encountered, such as rotting fruit. It has been reported that *C. elegans* are often found in such environments (Felix, 2007). It has already been suggested that the resistance of *C. elegans* to all volatile anaesthetics may have developed as a selective advantage,

due to their normal surroundings and permeability to simple organic compounds (Morgan and Sedensky, 1995).

This resistance could have many mechanisms. It is possible that the affinity of ethanol at key sensitive *C. elegans* proteins is lower when compared to their human homologs. Or the composition of the lipid membrane could be altered so as to reduce the partitioning of ethanol into the membrane and its access to its sites of action.

However as *C. elegans* shows a qualitatively similar response to ethanol to the human it can still be considered a good model for the effects of ethanol on humans. Concentrations of 10-100mM can be considered as equivalent to the intoxicating effects of ethanol, and concentrations of 100-300mM as equivalent to the sedative effects of ethanol.

By these definitions the concentration range of 250-350mM, which was used in Chapter 5 to condition worms to ethanol, falls at the outside edge of the sedative range. As was discussed in that chapter (see section 5.3.3) the development of alcohol dependence in humans is associated with repeated withdrawal which induces a kindling of the withdrawal response (Duka et al., 2004;Breese et al., 2005). Thus it would be interesting to investigate whether lower concentrations of ethanol could produce a withdrawal effect on *C. elegans* if administered and withdrawn repeatedly. However the clear development of tolerance and withdrawal described in Chapter 5 provides a useful, heuristic model for investigating the mechanisms by which neuroadaptation to ethanol occurs.

7.3 Distinguishing neuroadaptation from other chronic effects of ethanol exposure.

Chronic exposure to ethanol may have various effects in *C. elegans* which could not be considered to be neuroadaptation to ethanol. These include the developmental delay in response to chronic ethanol exposure demonstrated by Davis et al. (Davis et al., 2008), and reinforced by experiments in Chapter 5 showing a reduction in the size and egg laying ability of conditioned worms. Other chronic effects of ethanol could include an effect of ethanol on cellular stress pathways or, as a result of the reduction in pumping rate seen in acute intoxication, a food deprivation effect. Any of these chronic effects of ethanol may cause behavioural changes that persist after ethanol removal and could thus be confused with ethanol withdrawal. Although a cellular stress pathway has been described in *Drosophila* which contributes to tolerance to ethanol and so theoretically could also contribute to withdrawal (Scholz et al., 2005).

In this thesis a withdrawal relief effect has been shown in *C. elegans*. The behaviours of not reaching the food in the food race, increased unaccompanied omega turns, increased loopyness of locomotion and decreased efficiency and speed of locomotion can all be at least partially returned to basal levels by a low concentration of ethanol. This provides evidence that these are all a result of neuroadaptation to ethanol.

However the reduction in the rate of reversals, seen after ethanol conditioning is not returned to control levels by either a low or high concentration of ethanol. This is thus presumably due to one of the alternative effects of chronic ethanol exposure mentioned above. This demonstrates that neuroadaptation to ethanol can be distinguished from other potential effects of chronic ethanol in *C. elegans*.

7.4 *C. elegans* as a model for alcohol dependence.

As discussed in the introduction in one respect the use of *C. elegans* as a model for alcohol dependence is limited in that it cannot readily provide insight into the higher cognitive aspects of human addiction such as the development of compulsive use and relapse (Everitt et al., 2008;Rodd et al., 2004a;Stewart, 2008). However, it has been suggested that in humans the development of tolerance and dependence is underpinned by neuroadaptive processes (see (Koob and Le Moal, 2006) for review) and it has been shown in this study that it is possible to induce distinct ethanol-dependent behavioural states following prolonged exposure to ethanol, that are paradigms for the results of these neuroadaptive processes, in *C. elegans*.

In addition *C. elegans* have recently been shown to develop a preference for ethanol after chronic exposure (Lee et al., 2009). This may indicate that the adaptations revealed in withdrawal cause ethanol to be negatively reinforcing in *C. elegans* raising the possibility that *C. elegans* could be use to investigate the basis of the motivational aspects of the development of alcohol dependence.

C. elegans is then a useful system in which to study the entire process of this neuroadaptation, from the behaviour of the whole organism, through the circuits affecting this behaviour to the proteins on which ethanol is acting. A thorough understanding of how this can occur in *C. elegans* and other invertebrate models could go on to inform work in more complex organisms.

7.5 The mechanism of the development of alcohol dependence in *C. elegans.*

7.5.1 Neuropeptides

This study has shown that neuropeptides are required for the development of neuroadaptations leading to withdrawal from ethanol in *C. elegans*. However they do not appear to be required for intoxication. This makes it unlikely that neuropeptide release is a target of acute ethanol. Rather it seems likely sustained ethanol-induced signalling causes release (or inhibition of normal release) of neuropeptides which acts in a homeostatic manner to counter the effects of ethanol on the worm.

The majority of neuropeptides bind to G-protein coupled receptors (or tyrosine kinases in the case of the insulin-like peptides) and have a modulatory effect on synaptic transmission (Li and Kim, 2008). They may be released in response to higher neuronal firing frequencies or more sustained depolarisation than is required to release classical neurotransmitters (Heilig and Koob, 2007). This fits in with the results described here.

The *C. elegans* genome contains at least 113 neuropeptide genes encoding over 250 distinct neuropeptides (Li and Kim, 2008). These are expressed extensively throughout the nervous system and in non-neuronal tissues and have been implicated in many behaviours including locomotion, dauer formation, egg laying and social behaviour. In fact a recent review stated that neuropeptides are envisioned to be involved in all behaviours in *C. elegans* (Li and Kim, 2008). However the specific function of the majority of individual neuropeptides has yet to be elucidated. A first

step for further work would be to investigate which neuropeptides specifically were required for the development of withdrawal, and which neurons they were acting on to bring about these effects. It is quite possible that many different neuropeptide pathways are involved.

Many different neuropeptides have been implicated in the development of alcohol dependence in mammalian systems (see Chapter 1). These include the opioid peptides (Walker and Koob, 2008), neuropeptide Y (NPY) (Thorsell, 2007) and corticotrophin releasing factor (CRF) (Heilig and Koob, 2007), however with the exception of the NPY-receptor like neuropeptide receptors these do not have direct homologs in *C. elegans*. As described in the introduction, these are extensively involved in the development of negatively reinforcing withdrawal symptoms in mammalian systems and, with the exception of the μ-opioid receptor, are only involved in the acute effects of ethanol to a lesser extent.

One neuropeptide pathway that has been associated with the response to ethanol is that involving NPR-1. NPR-1 is a neuropeptide receptor with homology to the mammalian neuropeptide Y receptor. As was described in Chapter 5, a worm with a lower function allele of *npr-1* has been shown to display increased acute tolerance when measuring speed on agar plates (Davies et al., 2004a). In addition after a similar conditioning paradigm to the one used in this study, wild type (N2) worms removed from ethanol and placed on food plates have been shown to display social feeding behaviours (aggregating together in clumps on the edges of the bacterial lawn) despite normally being solitary feeders. This social feeding behaviour is a phenotype of loss of function mutations in *npr-1*. In contrast when worms with loss of function

mutations in *npr-1* were placed on acute ethanol plates they became solitary feeders (Davies et al., 2004a). Davies et al. thus proposed that acute ethanol activated the NPR-1 pathway downstream of NPR-1 and chronic ethanol thus caused a downregulation of this pathway.

However an *npr-1* loss of function mutant did not phenocopy ethanol withdrawal, or affect the development of ethanol withdrawal or relief from withdrawal in the food race. It can thus be inferred that withdrawal in the food race must be mediated by an alternative pathway. NPR-1 signalling would be expected to be much reduced in *egl-3* loss of function mutants due to a lack of peptide ligands to act on the receptor. However as, on food race plates, where *npr-1* loss of function mutants show normal ethanol withdrawal as measured by time to reach food, *egl-3* loss of function mutants do not show any sign of withdrawal as measured using the automated video analysis, it can be assumed that this phenotype is not due to the loss of NPR-1 signalling alone.

7.5.2 Serotonergic signalling

Serotonergic signalling is also implicated by the results described here as having a role in the acute and chronic effects of ethanol. This is because there are no significant effects of either intoxication or withdrawal on loopyness or efficiency of locomotion in worms which lack the ability to synthesize 5-HT. However effects of both intoxication and withdrawal are still detectable though reduced relative to controls, when measuring the speed of worms. This indicates that 5-HT signalling is not required for all effects of ethanol. Further work would be needed to resolve whether 5-HT has a role in the response to ethanol in *C. elegans*.

Were this to be confirmed, it would seem likely that, as 5-HT signalling affects both intoxication and withdrawal, it would be involved in the acute effect of ethanol. The effect of withdrawal would be expected to be reduced as a consequence of this. The fact that the worms lacking 5-HT synthesis appear to phenocopy the withdrawn worms to some extent, showing increased loopyness, might indicate that ethanol acts to stimulate either the release of 5-HT or possibly the actions of 5-HT on 5-HT receptors.

In mammalian systems acute ethanol increases 5-HT levels in many brain areas, such as the nucleus accumbens (Yoshimoto et al., 1992), central nucleus of the amygdala (Yoshimoto et al., 2000), hippocampus (Bare et al., 1998), caudate putamen (Thielen et al., 2001) and frontal cortex (Portas et al., 1994). However this is not necessarily an effect of increased activation of serotonergic neurons. One study showed that ethanol decreased firing rates of 5-HT neurons in the dorsal raphe nucleus whilst increasing 5-HT levels in the caudate putamen. This indicates that this rise in 5-HT levels must be a local effect of increased release from 5-HT terminals and/or decreased reuptake (Thielen et al., 2001).

It is possible that ethanol is acting in a similar manner to increase 5-HT levels in *C. elegans*. The gene *mod-5* encodes the Na^+ Cl^- dependent 5-HT transporter which is required for 5-HT uptake in *C. elegans*. This is orthologous to the human 5-HT transporter (Ranganathan et al., 2001). However whilst ethanol has been shown to inhibit clearance of 5-HT in mammalian systems, this has been shown to occur in a 5-HT transporter independent manner in the hippocampus (Daws et al., 2006). It would

still be interesting to investigate if ethanol was likely to be acting on this transporter to inhibit reuptake in *C. elegans*.

Another possibility is that ethanol is acting to increase the effect of 5-HT on one or more of its receptors. Ethanol has been shown to potentiate mammalian 5-HT_3 receptor function in neuroblastoma cells at concentrations of 25-100mM ethanol, which are relevant to intoxication *in vivo* (Lovinger, 1991). This has also been demonstrated in channels expressed in *Xenopus* oocytes (Machu and Harris, 1994).

However *C. elegans* does not have a direct homolog of the 5-HT_3 receptor. The 5-HT_3 receptor is the only ionotropic 5-HT receptor in mammalian systems and is a cation channel. *C. elegans* does have another ionotropic 5-HT receptor which has not been found in mammalian systems, the chloride channel MOD-1. This is similar to members of the nicotinic acetylcholine gated receptor family of ligand-gated ion channels, in particular to GABA and glycine gated chloride channels. The 5-HT_3 receptor is also a member of this family, many members of which have been described as targets for ethanol. MOD-1, however, is not blocked by 5-HT_{3A} specific antagonists. *C. elegans* with loss of function mutations in *mod-1* show resistance to paralysis by exogenous 5-HT. This indicates that were acute ethanol to stimulate MOD-1 it would reduce locomotion. MOD-1 is widely expressed in neurons of the head, ventral cord and tail (Ranganathan et al., 2000).

It would be very interesting if MOD-1 was shown to be an ethanol target, as it would provide further information as to the structural requirements for an ethanol sensitive protein. The search for alcohol and anaesthetic binding sites in members of this family

of ligand-gate ion channels is an area of active research, and many studies have used chimeric and single point mutated receptor constructs to analyse the structure/ pharmacology relationships for ethanol effects in these ethanol sensitive proteins (Hu et al., 2006;McBride et al., 2004).

Other 5-HT receptors that have been implicated in the ethanol response in mammalian systems, although not necessarily as direct targets are the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors. There are homologs of 5-HT_1 and 5-HT_2 receptors in *C. elegans*. These are SER-4 and SER-1 respectively. SER-1 is widely expressed including in ventral cord motor neurons, however SER-4 is only expressed in a few interneurons (Carnell et al., 2005;Carre-Pierrat et al., 2006). Further experiments could also investigate their role in the ethanol response in *C. elegans*.

It is interesting to note that *C. elegans* has been shown to be able to adapt to the effect of exogenous 5-HT. One effect of 5-HT is to initially stimulate egg-laying, an effect occurring through the SER-1 receptor. Wild type animals exposed to 5-HT overnight accumulated unlaid eggs, and were unable to lay eggs in response to a fresh dose of 5-HT. It was shown that the calcium channel subunit UNC-2 was required for this adaptation (Schafer and Kenyon, 1995).

It has also been shown that adapted worms that were transferred to plates without 5-HT exhibited a strong inhibition of egg laying after removal from 5-HT. This is a withdrawal effect. The animals recover from this effect in a few hours. This response does not occur in *ser-1* loss of function mutants indicating that it is dependent on the SER-1 receptor. In *ser-1* loss of function mutants there is a MOD-1 dependent

inhibition of egg laying. This MOD-1 dependent inhibition of egg laying by 5-HT diminishes over 4-5 hours chronic exposure to 5-HT indicating that *C. elegans* become tolerant to both the stimulatory and inhibitory effects of 5-HT on egg laying (Carnell et al., 2005).

It is possible that mechanisms similar to those which allow *C. elegans* to adapt to exogenous 5-HT might allow them to adapt to an ethanol stimulated increase in 5-HT signalling. However these experiments focus on the effect of 5-HT on egg laying, rather than locomotion, and these are distinct pathways. It is however interesting to speculate whether the effect of ethanol to decrease egg laying might be mediated through the MOD-1 dependent inhibitory pathway, were ethanol shown to have a stimulatory effect on MOD-1.

7.5.3 Dopaminergic signalling

As described in Chapter 6 the *cat-2 e1112* worms which have severely reduced dopamine levels appear to phenocopy some aspects of ethanol withdrawal, as the control *cat-2* worms are significantly loopier and less efficient than wild type, a pattern that is not repeated in the intoxicated or withdrawn worms.

However effects of both intoxication and withdrawal are still detectable when measuring the speed of worms. This may indicate that dopamine signalling is not required for all effects of ethanol. However *cat-2* worms still have approximately 40% of wild type dopamine levels, so it is possible that in the total absence of dopamine no withdrawal would be detected, for instance if the dopaminergic neurons were ablated. Further work would be needed to resolve the question of whether there is a role for dopamine in the response to ethanol in *C. elegans*.

Were this to be confirmed it would be possible that ethanol withdrawal would lead to decreased dopamine release or a reduction in signalling downstream of dopamine release but in the same pathway. Interestingly a major pathway affected by the G-protein coupled dopamine receptors DOP1-4 is cAMP signalling, which has been implicated in ethanol sensitivity in *Drosophila*. It has been shown that pathways downstream of dopaminergic signalling can adapt to continuous stimulation in *C. elegans* as tolerance to and withdrawal from exogenous dopamine have been previously described (Schafer and Kenyon, 1995). Interestingly these effects developed over a four hour period, a time frame similar to that seen for the development of neuroadaptation in this study.

In mammalian systems, ethanol increases dopamine release from the neurons of the mesolimbic dopamine pathway (see Chapter 1). This is due at least partially to a direct action on these neurons. The results described here do not appear to indicate however, that acute ethanol is stimulating dopamine release in *C. elegans* as in this case it would be expected that the effect of intoxication would be reduced in the *cat-2* worms. However, acute ethanol could be acting downstream of dopamine release to activate the same pathway.

One way it could do this is by an action on the dopamine receptors. A dopamine-gated chloride channel, LGC-53, which is part of the same nicotinic acetylcholine gated receptor family of receptors as MOD-1, has recently been described. It is conceivable that this receptor may well be sensitive to ethanol as many other members of this receptor family are ethanol sensitive. Deletion mutants of this receptor have been

described as showing no behavioural abnormalities; however it would be interesting to investigate their response to acute and chronic ethanol.

7.5.4 Other signalling pathways

This study has also provided evidence for potentially more minor roles for fast transmitters in the ethanol response. However further work would be needed to confirm or deny involvement of these signalling pathways.

One gene that has previously been described as being involved in the acute ethanol response is *slo-1*, which encodes that BK potassium channel. Loss of function mutations in this gene have been described as causing resistance to acute ethanol (Davies et al., 2003). The experiments described in Chapter 5 show a reduction in the effect of acute ethanol on speed on agar plates using the automated analysis, but not in several other assays. There is also no reduction in withdrawal behaviour. It would thus seem that SLO-1 has a more minor role in the ethanol effect than has previously been believed.

7.6 A model of the mechanism of neuroadaptation to ethanol in *C. elegans*

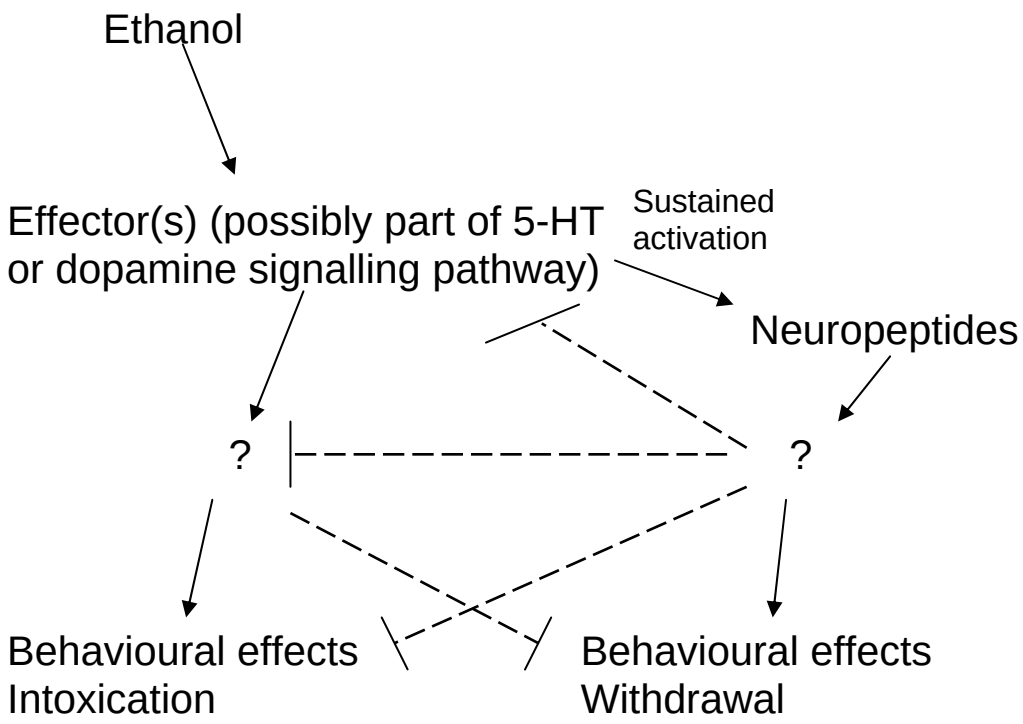


Figure 7.1 A model of the neuroadaptation to ethanol in *C. elegans*. In this model ethanol brings about its acute effects by acting on various effector proteins, possibly including part of the 5-HT or dopamine signalling pathways. Sustained activation of these proteins leads to the release of neuropeptides which act to counter the acute effects of ethanol, either by directly affecting the pathways containing the effector proteins or by acting elsewhere to bring about opposing behavioural effects. If ethanol is then removed these adaptations lead to withdrawal

7.7 The relevance of this study to alcoholism in humans.

In this study a paradigm for the investigation of the neuroadaptive processes that occur in response to chronic ethanol exposure in *C. elegans* has been developed. This study highlights the importance of neuropeptides in the neuroadaptive processes that can lead to the development of dependence. Many different neuropeptides have been implicated in the development of alcohol dependence in mammalian systems. These include the opioid peptides (Walker and Koob, 2008), neuropeptide Y (NPY)

(Thorsell, 2007) and corticotrophin releasing factor (CRF) (Heilig and Koob, 2007), however these do not have direct homologs in *C. elegans*. It is nevertheless possible that further study of the mechanism of neuropeptide-dependent neuroadaptation to ethanol in *C. elegans* may inform the understanding of the development of dependence in more complex organisms where in-depth study of a simple circuit is more difficult. This study also indicates a role for the neuromodulators dopamine and 5-HT in the ethanol response. This will provide a starting point a closer analysis of how ethanol can interact with these signalling pathways, illuminating how it might be acting in mammalian systems. In particular it would be interesting to investigate if ethanol could interact with the MOD-1, SER-1, SER-4 or LGC-53 receptors or with the MOD-5 serotonin transporter. Overall the development of a paradigm with which to study the neuroadaptation to ethanol in *C. elegans* will enable future studies to gain a precise understanding of how this process works from target proteins, through the circuits they act in, to the behaviours of the whole worm.

7.8 Conclusions

In conclusion this thesis has shown that *C. elegans* undergoes neuroadaptation to the chronic presence of ethanol, leading to tolerance to the presence of ethanol and withdrawal when ethanol is removed. This withdrawal behaviour can be shown to be the result of neuroadaptation as it is reduced by a low concentration of ethanol. Furthermore intoxication and withdrawal are distinct opposing behaviours which have been characterised using automated analysis of videos. Ethanol withdrawal has been shown to be neuropeptide dependent and there may be a role for 5-HT and dopamine in both the acute and chronic effects of ethanol.

Appendix A - A forward genetic screen

A forward genetic screen

In order to investigate the genetic basis of the neuroadaptation to ethanol in *C. elegans* a forward genetic screen for mutants defective in withdrawal behaviour was performed. Forward genetic screens enable the identification of relevant genes in a manner unbiased by previous work and expectations. Deficiency in withdrawal behaviour in the food race was screened for, as this was the most marked change in behaviour seen in response to neuroadaptation.

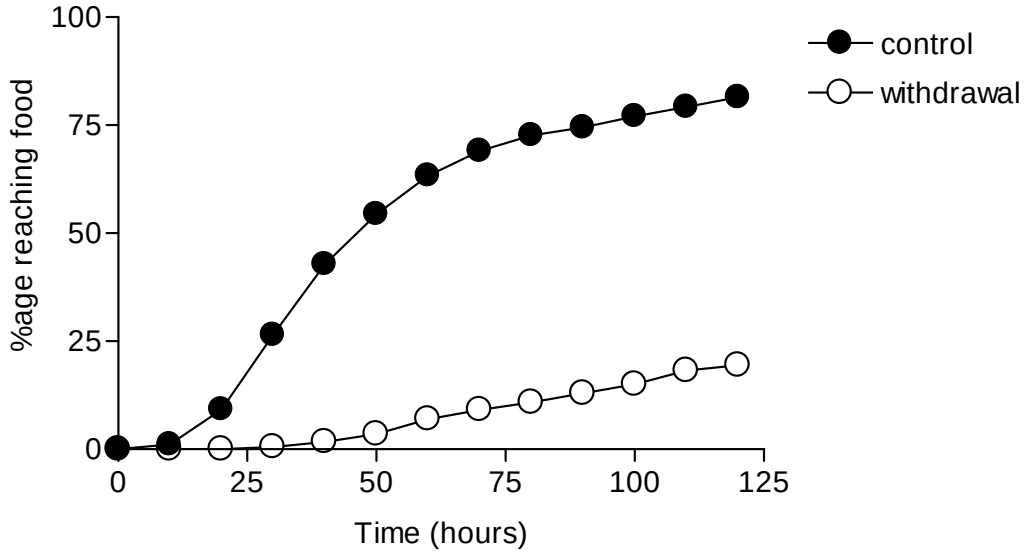


Figure A.1 Pooled data from 22 independent experiments showing the overall percentage of at least 750 worms per test condition reaching the food over a two hour period. Conditioning occurred for 48 hours at an average concentration of 282mM ethanol.

Data was pooled from all the food race experiments performed under the same conditions to calculate the percentage of the total worms that reached the food at each time point. This was used as an estimate of the probability of unmutagenised wild type worms reaching the food under these conditions (Figure A.1). Using this it was decided to screen for mutants that had reached the food at the 50 minute time point after 48 hours conditioning. At this time point 54.4% of control worms had reached the food but only 3.5% of withdrawn worms had. This was the point that maximised

the percentage of control worms reaching the food, and therefore maximised the rate of detection of true positives, without producing an unmanageable rate of false positives.

However, this meant that a high number of false positives could still be expected to come out of the screen. These would be worms that did not contain mutations relevant to the development of withdrawal but reached the food before the 50 minute time point anyway. 350 false positives could be expected for every 10,000 worms screened. It was therefore decided to grow up the progeny of any worms picked out of the initial screen and perform a population screen on these. In this any populations in which a sufficiently high percentage of the worms had not reached the food at the 50 minute time point would be discarded. Over 22 experiments the highest percentage of worms to have reached the food at the 50 minute time point was 20%. 25% was therefore set as the cut-off point for the population screen. This was to ensure that only worms containing a mutation that affected their ability to reach the food in the food race whilst under withdrawal-inducing conditions were picked out of the screen. To check that this was the case a practice screen was performed using the same method on non-mutagenised worms. No worms were picked out of this screen indicating that it successfully excluded false positives.

EMS mutagenesis was performed as described in chapter 2. 7500 haploid genomes were screened. Individual L4 worms that had survived the mutagenesis procedure were picked to individual plates. These were the F0 generation. These F0 worms were allowed to grow to adults and lay eggs for two nights before the F0 adults were removed. The eggs, which formed the F1 generation, were grown up and allowed to

self fertilise until they were gravid adults. They were then bleached to produce an age-synchronised F2 generation which could be grown up and screened for worms showing a reduction in withdrawal. As the F1 generation had been allowed to self fertilise, the F2 generation would have contained homozygous mutations. This allowed recessive mutations to be detected. It will also mean any homozygous mutations will be present in the progeny of the worm when it self fertilises.

When the age-synchronised F2 generation reached L4 they were washed onto ethanol plates and conditioned for 48 hours at 259mM ethanol. After 48 hours they were washed and placed onto food race plates. After 50 minutes on the food race plates any worms that had reached the food were picked onto individual plates. 175 worms were picked out of this stage of the screen. This was lower than expected from Figure A.1. This might indicate that a proportion of the worms had mutations that impaired locomotion in the food race.

These worms were allowed to self fertilise and lay eggs. These populations were grown up and bleached to produce an age synchronised population. When these age-synchronised populations reached L4 they were washed onto ethanol plates and conditioned for 48 hours at 215mM ethanol. After 48 hours they were washed and placed onto food race plates. The proportion of worms reaching the food at the 50 minute time point in each population was recorded.

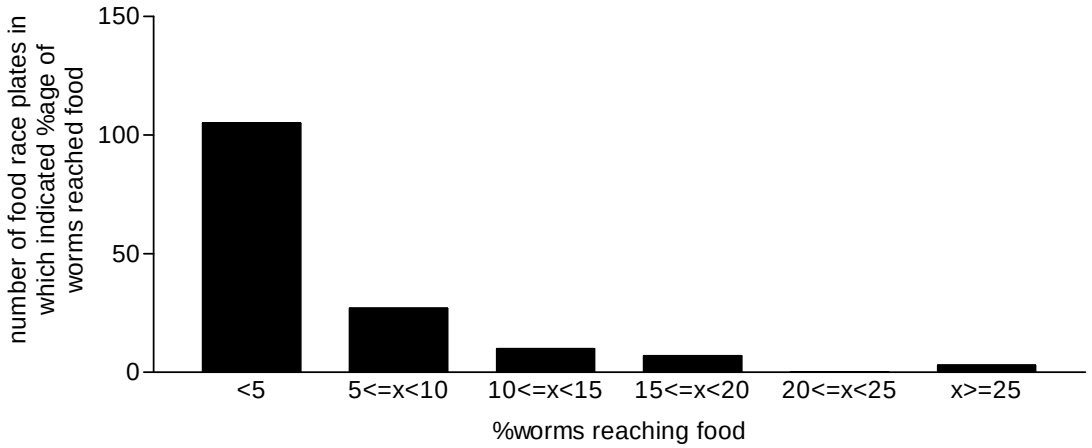


Figure A.2 Histogram showing the proportions of worms reaching the food on all the plates in the population screen

Three populations of worms were picked out of the population screen.

Originally from plate	Number worms reached food	Total worms	percentage worms reached food
33	23	92	25%
2	8	12	67%
85	29	52	56%

These populations of worms were tested in a full food race experiment to give a more detailed description of their behaviour.

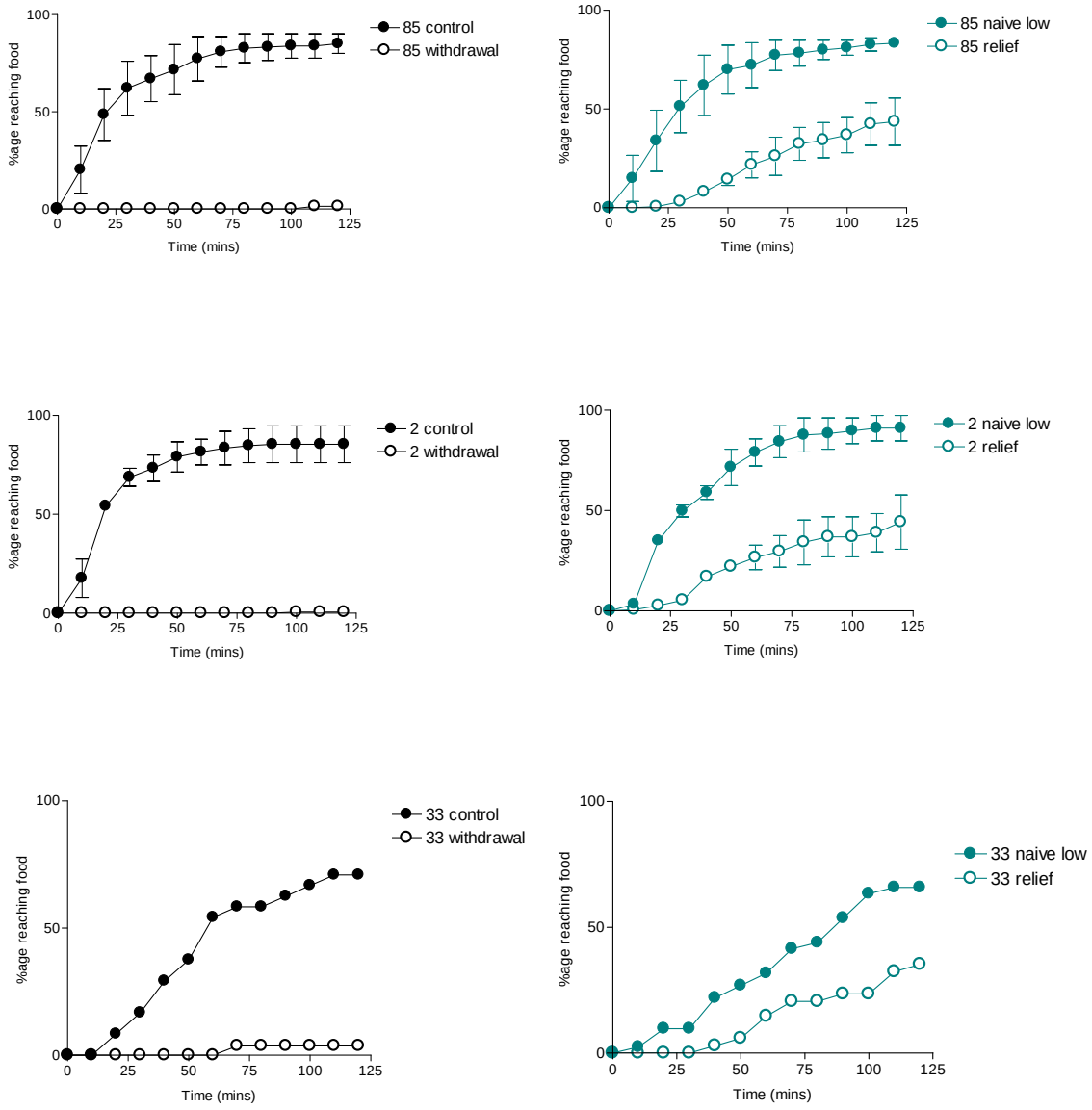


Figure A.3 The cumulative percentage of worms reaching the food every ten minutes on either 0mM ethanol (black) or 82mM ethanol (turquoise) food race plates. Withdrawal and relief worms (open circles) have been exposed to 218mM ethanol for 48 hours before the food race. Control and naive low worms (filled circles) have never been previously exposed to ethanol.

All three strains picked out of the screen showed normal withdrawal and relief behaviour in the food race. It was therefore concluded that they did not contain mutations that affected withdrawal behaviour in the food race and had been picked out of the screen in response to natural variation in behaviour.

This meant that the genetic screen had produced no strains containing mutations affecting withdrawal behaviour. One reason for this could be that the screen was not saturated; in fact a relatively low number of haploid genomes was screened due to the relatively high time required per genome to eliminate false positives. Therefore there could be single genes involved in withdrawal behaviour in the food race that the screen missed.

Another explanation could be that there are so many genes that are affected by ethanol withdrawal that none of them, individually, play a part large enough to have been detected by the screen. To be picked out of the screen a large change in behaviour would be required. If mutations in many different genes caused small effects, which, cumulatively, could cause a large effect, one would not expect to detect it.

This study therefore continued to investigate the genetic basis of the neuroadaptation effect by using a candidate gene approach to enable the detection of smaller changes in behaviour in the strains investigated.

Appendix B - Statistical analysis of data from the automated video analysis

Statistical analysis of data from videos

For each of seven mutant strains sets of videos were taken of them and matched N2 controls. The data produced is described in Chapter 4. In addition one set of videos of just N2 worms were taken during the initial experiments (Chapter 3). Each set of videos consisted of approximately 20 videos of worms under each of six conditions; control, naive low, naive high (intoxicated), withdrawal, relief and tolerance. From each video three aspects of the worms locomotion were measured by the automated video analysis. These were loopyness, efficiency and speed. This appendix shows the statistical analysis performed on these data sets using the program SPSS 15.0 in order to reach the conclusions described in Chapter 4.

Analysis for initial N2 results

One way ANOVA - Speed

Source	df	F	Sig.	p<0.05
condition	5	33.053	.000	y

One way ANOVA – Efficiency

Source	df	F	Sig.	p<0.05
condition	5	14.689	.000	y

One way ANOVA – Loopyness

Source	df	F	Sig.	p<0.05
condition	5	9.173	.000	y

Bonferroni Post-hoc tests - Speed

(I) condition	(J) condition	Sig.	p<0.05?
control	**naive low**	1.000	
	naive high	.000	y
	withdrawal	.000	y
	relief	.014	y
	tolerance	.000	y
naive low	**control**	1.000	
	naive high	.000	y
	withdrawal	.001	y
	relief	.120	
	tolerance	.000	y
naive high	**control**	.000	y
	naive low	.000	y
	withdrawal	.000	y
	relief	.000	y
	tolerance	1.000	
withdrawal	**control**	.000	y
	naive low	.001	y
	naive high	.000	y
	relief	1.000	
	tolerance	.000	y
relief	**control**	.014	y
	naive low	.120	
	naive high	.000	y
	withdrawal	1.000	
	tolerance	.000	y
tolerance	**control**	.000	y
	naive low	.000	y
	naive high	1.000	
	withdrawal	.000	y
	relief	.000	y

Bonferroni Post-hoc tests - Efficiency

(I) condition	(J) condition	Sig.	p<0.05?
control	**naive low**	1.000	
	naive high	.000	y
	withdrawal	.000	y
	relief	.288	
	tolerance	.009	y
naive low	**control**	1.000	
	naive high	.000	y
	withdrawal	.000	y
	relief	.055	
	tolerance	.001	y
naive high	**control**	.000	y
	naive low	.000	y
	withdrawal	1.000	
	relief	.004	y
	tolerance	.129	
withdrawal	**control**	.000	y
	naive low	.000	y
	naive high	1.000	
	relief	.218	
	tolerance	1.000	
relief	**control**	.288	
	naive low	.055	
	naive high	.004	y
	withdrawal	.218	
	tolerance	1.000	
tolerance	**control**	.009	y
	naive low	.001	y
	naive high	.129	
	withdrawal	1.000	
	relief	1.000	

Bonferroni Post-hoc tests - Loopyness

(I) condition	(J) condition	Sig.	p<0.05?
control	**naive low**	1.000	
	naive high	1.000	
	withdrawal	.000	y
	relief	.788	
	tolerance	1.000	
naive low	**control**	1.000	
	naive high	1.000	
	withdrawal	.000	y
	relief	.080	
	tolerance	1.000	
naive high	**control**	1.000	
	naive low	1.000	
	withdrawal	.000	y
	relief	.800	
	tolerance	1.000	
withdrawal	**control**	.000	
	naive low	.000	
	naive high	.000	y
	relief	.126	
	tolerance	.000	
relief	**control**	.788	
	naive low	.080	
	naive high	.800	
	withdrawal	.126	
	tolerance	.200	
tolerance	**control**	1.000	
	naive low	1.000	
	naive high	1.000	
	withdrawal	.000	y
	relief	.200	

Analysis for *egl-3* - Speed

Two way ANOVA

Source	df	F	Sig.	p<0.05
genotype	1	9.570	.002	y
condition	5	72.201	.000	y
genotype * condition	5	9.156	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05
N2	**condition**	5	50.806	.000	y
egl-3	**condition**	5	34.628	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05
control	**genotype**	1	1.226	.275	
naive low	**genotype**	1	6.361	.016	y
naive high	**genotype**	1	5.338	.026	y
withdrawal	**genotype**	1	8.946	.005	y
relief	**genotype**	1	18.039	.000	y
tolerance	**genotype**	1	.016	.900	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	control	naive low	1.000		*egl-3*	control	naive low	1.000	
		naive high	.000	y			naive high	.000	y
		withdrawal	.000	y			withdrawal	1.000	
		relief	.000	y			relief	.351	
		tolerance	.000	y			tolerance	.000	y
	naive low	control	1.000			naive low	control	1.000	
		naive high	.000	y			naive high	.000	y
		withdrawal	.000	y			withdrawal	1.000	
		relief	.000	y			relief	.022	y
		tolerance	.000	y			tolerance	.000	y
	naive high	control	.000	y		naive high	control	.000	y
		naive low	.000	y			naive low	.000	y
		withdrawal	.000	y			withdrawal	.000	y
		relief	.000	y			relief	.000	y
		tolerance	1.000				tolerance	1.000	
	withdrawal	control	.000	y		withdrawal	control	1.000	
		naive low	.000	y			naive low	1.000	
		naive high	.000	y			naive high	.000	y
		relief	1.000				relief	1.000	
		tolerance	.000	y			tolerance	.000	y
	relief	control	.000	y		relief	control	.351	
		naive low	.000	y			naive low	.022	y
		naive high	.000	y			naive high	.000	y
		withdrawal	1.000				withdrawal	1.000	
		tolerance	.000	y			tolerance	.000	y
	tolerance	control	.000	y		tolerance	control	.000	y
		naive low	.000	y			naive low	.000	y
		naive high	1.000				naive high	1.000	
		withdrawal	.000	y			withdrawal	.000	y
		relief	.000	y			relief	.000	y

Analysis for *egl-3* - Efficiency

Two way ANOVA

Source	df	F	Sig.	p<0.05
genotype	1	14.094	.000	y
condition	5	36.223	.000	y
genotype * condition	5	13.776	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05
N2	**condition**	5	33.633	.000	y
egl-3	**condition**	5	16.444	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	5.476	.025	y
naive low	**genotype**	1	10.419	.003	y
naive high	**genotype**	1	2.390	.130	
withdrawal	**genotype**	1	31.169	.000	y
relief	**genotype**	1	27.241	.000	y
tolerance	**genotype**	1	.005	.942	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***egl-3***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	1.000	
		relief	.000	y			**relief**	.982	
		tolerance	.000	y			**tolerance**	.078	
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	1.000	
		relief	.000	y			**relief**	1.000	
		tolerance	.000	y			**tolerance**	.026	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	1.000				**withdrawal**	.000	y
		relief	.000	y			**relief**	.000	y
		tolerance	.000	y			**tolerance**	.052	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	1.000	
		naive low	.000	y			**naive low**	1.000	
		naive high	1.000				**naive high**	.000	y
		relief	.136				**relief**	1.000	
		tolerance	.042	y			**tolerance**	.004	y
	relief	**control**	.000	y		**relief**	**control**	.982	
		naive low	.000	y			**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.136				**withdrawal**	1.000	
		tolerance	1.000				**tolerance**	.000	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.078	
		naive low	.000	y			**naive low**	.026	y
		naive high	.000	y			**naive high**	.052	
		withdrawal	.042	y			**withdrawal**	.004	y
		relief	1.000				**relief**	.000	y

Analysis for *egl-3* - Loopyness

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	2.401	.123	
condition	5	9.183	.000	y
genotype * condition	5	12.315	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	22.011	.000	y
egl-3	**condition**	5	.579	.716	

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	14.420	.001	y
naive low	**genotype**	1	11.058	.002	y
naive high	**genotype**	1	14.677	.000	y
withdrawal	**genotype**	1	15.065	.000	y
relief	**genotype**	1	4.411	.043	y
tolerance	**genotype**	1	3.632	.064	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	control	naive low	1.000		*egl-3*	control	naive low	1.000	
		naive high	1.000				naive high	1.000	
		withdrawal	.000	y			withdrawal	1.000	
		relief	.012	y			relief	1.000	
		tolerance	1.000				tolerance	1.000	
	naive low	control	1.000			naive low	control	1.000	
		naive high	1.000				naive high	1.000	
		withdrawal	.000	y			withdrawal	1.000	
		relief	.001	y			relief	1.000	
		tolerance	1.000				tolerance	1.000	
	naive high	control	1.000			naive high	control	1.000	
		naive low	1.000				naive low	1.000	
		withdrawal	.000	y			withdrawal	1.000	
		relief	.000	y			relief	1.000	
		tolerance	1.000				tolerance	1.000	
	withdrawal	control	.000	y		withdrawal	control	1.000	
		naive low	.000	y			naive low	1.000	
		naive high	.000	y			naive high	1.000	
		relief	.006	y			relief	1.000	
		tolerance	.000	y			tolerance	1.000	
	relief	control	.012	y		relief	control	1.000	
		naive low	.001	y			naive low	1.000	
		naive high	.000	y			naive high	1.000	
		withdrawal	.006	y			withdrawal	1.000	
		tolerance	.001	y			tolerance	1.000	
	tolerance	control	1.000			tolerance	control	1.000	
		naive low	1.000				naive low	1.000	
		naive high	1.000				naive high	1.000	
		withdrawal	.000	y			withdrawal	1.000	
		relief	.001	y			relief	1.000	

Analysis for *tph-1* - Speed

Two way ANOVA

Source	df	F	Sig.	p<0.05
genotype	1	160.174	.000	y
condition	5	35.991	.000	y
genotype * condition	5	9.334	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05
N2	**condition**	5	27.518	.000	y
tph-1	**condition**	5	8.481	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05
control	**genotype**	1	73.217	.000	y
naive low	**genotype**	1	55.907	.000	y
naive high	**genotype**	1	2.485	.124	
withdrawal	**genotype**	1	29.289	.000	y
relief	**genotype**	1	22.622	.000	y
tolerance	**genotype**	1	13.873	.001	y

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***tph-1***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.001	y
		withdrawal	.000	y			**withdrawal**	.035	y
		relief	.000	y			**relief**	.290	
		tolerance	.000	y			**tolerance**	.000	y
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.015	y
		withdrawal	.000	y			**withdrawal**	.249	
		relief	.001	y			**relief**	1.000	
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.001	y
		naive low	.000	y			**naive low**	.015	y
		withdrawal	.074				**withdrawal**	1.000	
		relief	.016	y			**relief**	1.000	
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.035	y
		naive low	.000	y			**naive low**	.249	
		naive high	.074				**naive high**	1.000	
		relief	1.000				**relief**	1.000	
		tolerance	.005	y			**tolerance**	.430	
	relief	**control**	.000	y		**relief**	**control**	.290	
		naive low	.001	y			**naive low**	1.000	
		naive high	.016	y			**naive high**	1.000	
		withdrawal	1.000				**withdrawal**	1.000	
		tolerance	.001	y			**tolerance**	.036	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	1.000	
		withdrawal	.005	y			**withdrawal**	.430	
		relief	.001	y			**relief**	.036	y

Analysis for *tph-1* - Efficiency

Two way ANOVA

Source	df	F	Sig.	p<0.05
genotype	1	14.776	.000	y
condition	5	23.224	.000	y
genotype * condition	5	5.504	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05
N2	**condition**	5	29.938	.000	y
tph-1	**condition**	5	4.018	.002	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	19.060	.000	y
naive low	**genotype**	1	8.713	.005	y
naive high	**genotype**	1	.688	.412	
withdrawal	**genotype**	1	4.635	.038	y
relief	**genotype**	1	5.774	.021	y
tolerance	**genotype**	1	5.944	.020	y

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05
N2	**control**	**naive low**	1.000		***tph-1***	**control**	**naive low**	.471	
		naive high	.000	y			**naive high**	.712	
		withdrawal	.005	y			**withdrawal**	1.000	
		relief	1.000				**relief**	1.000	
		tolerance	.000	y			**tolerance**	1.000	
	naive low	**control**	1.000			**naive low**	**control**	.471	
		naive high	.000	y			**naive high**	.001	y
		withdrawal	.000	y			**withdrawal**	.016	y
		relief	.093				**relief**	.228	
		tolerance	.000	y			**tolerance**	.064	
	naive high	**control**	.000	y		**naive high**	**control**	.712	
		naive low	.000	y			**naive low**	.001	y
		withdrawal	.001	y			**withdrawal**	1.000	
		relief	.000	y			**relief**	1.000	
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.005	y		**withdrawal**	**control**	1.000	
		naive low	.000	y			**naive low**	.016	y
		naive high	.001	y			**naive high**	1.000	
		relief	.259				**relief**	1.000	
		tolerance	.004	y			**tolerance**	1.000	
	relief	**control**	1.000			**relief**	**control**	1.000	
		naive low	.093				**naive low**	.228	
		naive high	.000	y			**naive high**	1.000	
		withdrawal	.259				**withdrawal**	1.000	
		tolerance	.000	y			**tolerance**	1.000	
	tolerance	**control**	.000	y		**tolerance**	**control**	1.000	
		naive low	.000	y			**naive low**	.064	
		naive high	1.000				**naive high**	1.000	
		withdrawal	.004	y			**withdrawal**	1.000	
		relief	.000	y			**relief**	1.000	

Analysis for *tph-1* - Loopyness

Two way ANOVA

Source	df	F	Sig.	p<0.05
genotype	1	13.106	.000	y
condition	5	10.144	.000	y
genotype * condition	5	1.413	.220	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	7.059	.000	y
tph-1	**condition**	5	5.185	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	13.696	.001	y
naive low	**genotype**	1	4.275	.046	
naive high	**genotype**	1	2.389	.131	
withdrawal	**genotype**	1	.157	.694	
relief	**genotype**	1	5.134	.029	y
tolerance	**genotype**	1	.082	.776	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05 ?	genotype	(I) condition	(J) condition	Sig.	p<0.05 ?
N2	control	naive low	.730		*tph-1*	control	naive low	.077	
		naive high	1.000				naive high	1.000	
		withdrawal	.005	y			withdrawal	1.000	
		relief	1.000				relief	1.000	
		tolerance	1.000				tolerance	.020	y
	naive low	control	.730			naive low	control	.077	
		naive high	.487				naive high	.667	
		withdrawal	.000	y			withdrawal	.013	y
		relief	.252				relief	.094	
		tolerance	1.000				tolerance	1.000	
	naive high	control	1.000			naive high	control	1.000	
		naive low	.487				naive low	.667	
		withdrawal	.015	y			withdrawal	1.000	
		relief	1.000				relief	1.000	
		tolerance	1.000				tolerance	.234	
	withdrawal	control	.005	y		withdrawal	control	1.000	
		naive low	.000	y			naive low	.013	y
		naive high	.015	y			naive high	1.000	
		relief	.028	y			relief	1.000	
		tolerance	.001	y			tolerance	.003	y
	relief	control	1.000			relief	control	1.000	
		naive low	.252				naive low	.094	
		naive high	1.000				naive high	1.000	
		withdrawal	.028	y			withdrawal	1.000	
		tolerance	1.000				tolerance	.025	y
	tolerance	control	1.000			tolerance	control	.020	y
		naive low	1.000				naive low	1.000	
		naive high	1.000				naive high	.234	
		withdrawal	.001	y			withdrawal	.003	y
		relief	1.000				relief	.025	y

Analysis for *cat-2* - Speed

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	4.242	.041	y
condition	5	52.302	.000	y
genotype * condition	5	.416	.837	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	29.777	.000	y
cat-2	**condition**	5	23.027	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	1.846	.183	
naive low	**genotype**	1	1.706	.200	
naive high	**genotype**	1	.449	.507	
withdrawal	**genotype**	1	1.623	.211	
relief	**genotype**	1	.008	.928	
tolerance	**genotype**	1	.029	.867	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***cat-2***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.001	y			**withdrawal**	.001	y
		relief	.000	y			**relief**	.037	y
		tolerance	.000	y			**tolerance**	.000	y
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.005	y			**withdrawal**	.004	y
		relief	.002	y			**relief**	.145	
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	.001	y			**withdrawal**	.008	y
		relief	.001	y			**relief**	.000	y
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.001	y		**withdrawal**	**control**	.001	y
		naive low	.005	y			**naive low**	.004	y
		naive high	.001	y			**naive high**	.008	y
		relief	1.000				**relief**	1.000	
		tolerance	.000	y			**tolerance**	.005	y
	relief	**control**	.000	y		**relief**	**control**	.037	y
		naive low	.002	y			**naive low**	.145	
		naive high	.001	y			**naive high**	.000	y
		withdrawal	1.000				**withdrawal**	1.000	
		tolerance	.000	y			**tolerance**	.000	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	1.000	
		withdrawal	.000	y			**withdrawal**	.005	y
		relief	.000	y			**relief**	.000	y

Analysis for *cat-2* - Efficiency

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	.001	.980	
condition	5	23.368	.000	y
genotype * condition	5	1.946	.088	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	20.506	.000	y
cat-2	**condition**	5	6.531	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	4.482	.041	y
naive low	**genotype**	1	7.547	.009	y
naive high	**genotype**	1	1.095	.302	
withdrawal	**genotype**	1	.309	.582	
relief	**genotype**	1	1.350	.252	
tolerance	**genotype**	1	.341	.563	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***cat-2***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.028	y
		withdrawal	.000	y			**withdrawal**	.214	
		relief	.198				**relief**	1.000	
		tolerance	.000	y			**tolerance**	.057	y
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.003	y
		withdrawal	.000	y			**withdrawal**	.031	y
		relief	.008	y			**relief**	1.000	
		tolerance	.000	y			**tolerance**	.006	y
	naive high	**control**	.000	y		**naive high**	**control**	.028	y
		naive low	.000	y			**naive low**	.003	y
		withdrawal	1.000				**withdrawal**	1.000	
		relief	.001	y			**relief**	.006	y
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.214	
		naive low	.000	y			**naive low**	.031	y
		naive high	1.000				**naive high**	1.000	
		relief	.188				**relief**	.061	
		tolerance	1.000				**tolerance**	1.000	
	relief	**control**	.198			**relief**	**control**	1.000	
		naive low	.008	y			**naive low**	1.000	
		naive high	.001	y			**naive high**	.006	y
		withdrawal	.188				**withdrawal**	.061	
		tolerance	.010	y			**tolerance**	.014	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.057	
		naive low	.000	y			**naive low**	.006	y
		naive high	1.000				**naive high**	1.000	
		withdrawal	1.000				**withdrawal**	1.000	
		relief	.010	y			**relief**	.014	y

Analysis for *cat-2* - Loopyness

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	1.156	.283	
condition	5	23.163	.000	y
genotype * condition	5	2.957	.013	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	17.168	.000	y
cat-2	**condition**	5	8.949	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	5.391	.026	y
naive low	**genotype**	1	10.015	.003	y
naive high	**genotype**	1	.924	.343	
withdrawal	**genotype**	1	1.673	.204	
relief	**genotype**	1	.697	.409	
tolerance	**genotype**	1	1.423	.240	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***cat-2***	**control**	**naive low**	1.000	
		naive high	1.000				**naive high**	.003	y
		withdrawal	.000	y			**withdrawal**	.200	
		relief	.122				**relief**	1.000	
		tolerance	1.000				**tolerance**	1.000	
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	1.000				**naive high**	.048	y
		withdrawal	.000	y			**withdrawal**	.011	y
		relief	.004	y			**relief**	1.000	
		tolerance	1.000				**tolerance**	1.000	
	naive high	**control**	1.000			**naive high**	**control**	.003	y
		naive low	1.000				**naive low**	.048	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.006	y			**relief**	.009	y
		tolerance	1.000				**tolerance**	.155	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.200	
		naive low	.000	y			**naive low**	.011	y
		naive high	.000	y			**naive high**	.000	y
		relief	.004	y			**relief**	.046	y
		tolerance	.000	y			**tolerance**	.002	y
	relief	**control**	.122			**relief**	**control**	1.000	
		naive low	.004	y			**naive low**	1.000	
		naive high	.006	y			**naive high**	.009	y
		withdrawal	.004	y			**withdrawal**	.046	y
		tolerance	.027	y			**tolerance**	1.000	
	tolerance	**control**	1.000			**tolerance**	**control**	1.000	
		naive low	1.000				**naive low**	1.000	
		naive high	1.000				**naive high**	.155	
		withdrawal	.000	y			**withdrawal**	.002	y
		relief	.027	y			**relief**	1.000	

Analysis for *unc-25* - Speed

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	124.089	.000	y
condition	5	45.333	.000	y
genotype * condition	5	8.230	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	31.021	.000	y
unc-25	**condition**	5	17.665	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	56.189	.000	y
naive low	**genotype**	1	23.047	.000	y
naive high	**genotype**	1	2.160	.150	
withdrawal	**genotype**	1	28.562	.000	y
relief	**genotype**	1	32.722	.000	y
tolerance	**genotype**	1	1.307	.261	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***unc-25***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.031	y			**withdrawal**	.000	y
		relief	.199				**relief**	.000	y
		tolerance	.000	y			**tolerance**	.000	y
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.087				**withdrawal**	.001	y
		relief	.460				**relief**	.005	y
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	.000	y			**withdrawal**	.505	
		relief	.000	y			**relief**	.169	
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.031	y		**withdrawal**	**control**	.000	y
		naive low	.087				**naive low**	.001	y
		naive high	.000	y			**naive high**	.505	
		relief	1.000				**relief**	1.000	
		tolerance	.000	y			**tolerance**	1.000	
	relief	**control**	.199			**relief**	**control**	.000	y
		naive low	.460				**naive low**	.005	y
		naive high	.000	y			**naive high**	.169	
		withdrawal	1.000				**withdrawal**	1.000	
		tolerance	.000	y			**tolerance**	.726	
	tolerance	**control**	.000	y		**tolerance**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	1.000	
		withdrawal	.000	y			**withdrawal**	1.000	
		relief	.000				**relief**	.726	

Analysis for *unc-25* - Efficiency

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	7.548	.007	y
condition	5	22.293	.000	y
genotype * condition	5	4.232	.001	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	24.517	.000	y
unc-25	**condition**	5	7.620	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05
control	**genotype**	1	2.021	.164	
naive low	**genotype**	1	.010	.921	
naive high	**genotype**	1	22.270	.000	y
withdrawal	**genotype**	1	2.557	.119	
relief	**genotype**	1	.136	.714	
tolerance	**genotype**	1	7.340	.010	y

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***unc-25***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.012	y
		withdrawal	.004	y			**withdrawal**	.000	y
		relief	1.000				**relief**	.597	
		tolerance	.001	y			**tolerance**	1.000	
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.017	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.438				**relief**	.775	
		tolerance	.000	y			**tolerance**	1.000	
	naive high	**control**	.000	y		**naive high**	**control**	.012	y
		naive low	.000	y			**naive low**	.017	y
		withdrawal	.000	y			**withdrawal**	1.000	
		relief	.000	y			**relief**	1.000	
		tolerance	.001	y			**tolerance**	1.000	
	withdrawal	**control**	.004	y		**withdrawal**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	.000	y			**naive high**	1.000	
		relief	.064				**relief**	.066	
		tolerance	1.000				**tolerance**	.023	y
	relief	**control**	1.000			**relief**	**control**	.597	
		naive low	.438				**naive low**	.775	
		naive high	.000	y			**naive high**	1.000	
		withdrawal	.064				**withdrawal**	.066	
		tolerance	.016	y			**tolerance**	1.000	
	tolerance	**control**	.001	y		**tolerance**	**control**	1.000	
		naive low	.000	y			**naive low**	1.000	
		naive high	.001	y			**naive high**	1.000	
		withdrawal	1.000				**withdrawal**	.023	y
		relief	.016	y			**relief**	1.000	

Analysis for *unc-25* - Loopyness

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	6.301	.013	y
condition	5	14.892	.000	y
genotype * condition	5	1.123	.349	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	9.899	.000	y
unc-25	**condition**	5	6.470	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05
control	**genotype**	1	.196	.660	
naive low	**genotype**	1	.050	.825	
naive high	**genotype**	1	7.214	.011	y
withdrawal	**genotype**	1	1.191	.282	
relief	**genotype**	1	2.119	.154	
tolerance	**genotype**	1	.000	.986	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***unc-25***	**control**	**naive low**	1.000	
		naive high	1.000				**naive high**	.564	
		withdrawal	.002	y			**withdrawal**	.105	
		relief	.892				**relief**	1.000	
		tolerance	.162				**tolerance**	.858	
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	1.000				**naive high**	1.000	
		withdrawal	.000	y			**withdrawal**	.033	y
		relief	.259				**relief**	1.000	
		tolerance	.800				**tolerance**	1.000	
	naive high	**control**	1.000			**naive high**	**control**	.564	
		naive low	1.000				**naive low**	1.000	
		withdrawal	.005	y			**withdrawal**	.000	y
		relief	1.000				**relief**	.082	
		tolerance	.074				**tolerance**	1.000	
	withdrawal	**control**	.002	y		**withdrawal**	**control**	.105	
		naive low	.000	y			**naive low**	.033	y
		naive high	.005	y			**naive high**	.000	y
		relief	.635				**relief**	.799	
		tolerance	.000	y			**tolerance**	.000	y
	relief	**control**	.892			**relief**	**control**	1.000	
		naive low	.259				**naive low**	1.000	
		naive high	1.000				**naive high**	.082	
		withdrawal	.635				**withdrawal**	.799	
		tolerance	.000	y			**tolerance**	.141	
	tolerance	**control**	.162			**tolerance**	**control**	.858	
		naive low	.800				**naive low**	1.000	
		naive high	.074				**naive high**	1.000	
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.000	y			**relief**	.141	

Analysis for *unc-49* - Speed

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	59.327	.000	y
condition	5	24.872	.000	y
genotype * condition	5	.928	.463	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	11.062	.000	y
unc-49	**condition**	5	17.776	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	15.305	.000	y
naive low	**genotype**	1	7.189	.011	y
naive high	**genotype**	1	4.933	.033	y
withdrawal	**genotype**	1	28.019	.000	y
relief	**genotype**	1	19.121	.000	y
tolerance	**genotype**	1	2.284	.140	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***unc-49***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.827				**withdrawal**	.001	y
		relief	.282				**relief**	.008	y
		tolerance	.000	y			**tolerance**	.000	y
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	1.000				**withdrawal**	.003	y
		relief	1.000				**relief**	.034	y
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	.025	y			**withdrawal**	.100	
		relief	.063				**relief**	.010	y
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.827			**withdrawal**	**control**	.001	y
		naive low	1.000				**naive low**	.003	y
		naive high	.025	y			**naive high**	.100	
		relief	1.000				**relief**	1.000	
		tolerance	.004	y			**tolerance**	.269	
	relief	**control**	.282			**relief**	**control**	.008	y
		naive low	1.000				**naive low**	.034	y
		naive high	.063				**naive high**	.010	y
		withdrawal	1.000				**withdrawal**	1.000	
		tolerance	.011	y			**tolerance**	.033	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	1.000	
		withdrawal	.004	y			**withdrawal**	.269	
		relief	.011	y			**relief**	.033	y

Analysis for *unc-49* - Efficiency

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	.642	.424	
condition	5	26.682	.000	y
genotype * condition	5	1.404	.224	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	13.421	.000	y
unc-49	**condition**	5	14.671	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	.493	.487	
naive low	**genotype**	1	4.770	.035	y
naive high	**genotype**	1	.242	.626	
withdrawal	**genotype**	1	.919	.344	
relief	**genotype**	1	.692	.411	
tolerance	**genotype**	1	2.515	.122	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***unc-49***	**control**	**naive low**	.350	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.014	y			**relief**	1.000	
		tolerance	.000	y			**tolerance**	.250	
	naive low	**control**	1.000			**naive low**	**control**	.350	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.008	y			**relief**	.003	y
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	1.000				**withdrawal**	1.000	
		relief	.583				**relief**	.025	y
		tolerance	1.000				**tolerance**	.393	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	1.000	
		relief	1.000				**relief**	.060	
		tolerance	1.000				**tolerance**	.787	
	relief	**control**	.014	y		**relief**	**control**	1.000	
		naive low	.008	y			**naive low**	.003	y
		naive high	.583				**naive high**	.025	y
		withdrawal	1.000				**withdrawal**	.060	
		tolerance	.910				**tolerance**	1.000	
	tolerance	**control**	.000	y		**tolerance**	**control**	.250	
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	.393	
		withdrawal	1.000				**withdrawal**	.787	
		relief	.910				**relief**	1.000	

Analysis for *unc-49* - Loopyness

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	.500	.480	
condition	5	24.262	.000	y
genotype * condition	5	1.453	.206	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	17.624	.000	y
unc-49	**condition**	5	10.430	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	2.718	.107	
naive low	**genotype**	1	.955	.335	
naive high	**genotype**	1	1.657	.206	
withdrawal	**genotype**	1	.076	.784	
relief	**genotype**	1	.168	.684	
tolerance	**genotype**	1	1.783	.190	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	control	naive low	1.000		*unc-49*	control	naive low	.113	
		naive high	1.000				naive high	1.000	
		withdrawal	.000	y			withdrawal	.007	y
		relief	.002	y			relief	.868	
		tolerance	1.000				tolerance	1.000	
	naive low	control	1.000			naive low	control	.113	
		naive high	1.000				naive high	.444	
		withdrawal	.000	y			withdrawal	.000	y
		relief	.000	y			relief	.000	y
		tolerance	.146				tolerance	1.000	
	naive high	control	1.000			naive high	control	1.000	
		naive low	1.000				naive low	.444	
		withdrawal	.000	y			withdrawal	.001	y
		relief	.000	y			relief	.290	
		tolerance	.218				tolerance	1.000	
	withdrawal	control	.000	y		withdrawal	control	.007	y
		naive low	.000	y			naive low	.000	y
		naive high	.000	y			naive high	.001	y
		relief	.232				relief	1.000	
		tolerance	.000	y			tolerance	.000	y
	relief	control	.002	y		relief	control	.868	
		naive low	.000	y			naive low	.000	y
		naive high	.000	y			naive high	.290	
		withdrawal	.232				withdrawal	1.000	
		tolerance	.243				tolerance	.019	y
	tolerance	control	1.000			tolerance	control	1.000	
		naive low	.146				naive low	1.000	
		naive high	.218				naive high	1.000	
		withdrawal	.000	y			withdrawal	.000	y
		relief	.243				relief	.019	y

Analysis for *eat-4* - Speed

Two way ANOVA

Source	df	F	Sig.	p<0.05?
condition	5	75.221	.000	y
genotype	1	108.347	.000	y
condition * genotype	5	1.907	.094	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	35.989	.000	y
eat-4	**condition**	5	44.077	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	46.862	.000	y
naive low	**genotype**	1	7.304	.010	y
naive high	**genotype**	1	32.573	.000	y
withdrawal	**genotype**	1	40.573	.000	y
relief	**genotype**	1	25.121	.000	y
tolerance	**genotype**	1	5.124	.030	y

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***eat-4***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.000	y			**relief**	.000	y
		tolerance	.000	y			**tolerance**	.000	y
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.014	y			**withdrawal**	.000	y
		relief	.009	y			**relief**	.000	y
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.000	y			**relief**	.000	y
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.000	y
		naive low	.014	y			**naive low**	.000	y
		naive high	.000	y			**naive high**	.000	y
		relief	1.000				**relief**	1.000	
		tolerance	.000	y			**tolerance**	.001	y
	relief	**control**	.000	y		**relief**	**control**	.000	y
		naive low	.009	y			**naive low**	.000	y
		naive high	.000	y			**naive high**	.000	y
		withdrawal	1.000				**withdrawal**	1.000	
		tolerance	.000	y			**tolerance**	.000	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	1.000	
		withdrawal	.000	y			**withdrawal**	.001	y
		relief	.000	y			**relief**	.000	y

Analysis for *eat-4* - Efficiency

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	.003	.954	
condition	5	44.109	.000	y
genotype * condition	5	4.085	.001	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05
N2	**condition**	5	21.864	.000	y
eat-4	**condition**	5	26.491	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	1.262	.269	
naive low	**genotype**	1	1.820	.186	
naive high	**genotype**	1	1.323	.258	
withdrawal	**genotype**	1	2.983	.093	
relief	**genotype**	1	.701	.408	
tolerance	**genotype**	1	12.783	.001	y

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***eat-4***	**control**	**naive low**	.328	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.047	y			**relief**	.071	
		tolerance	.000	y			**tolerance**	.225	
	naive low	**control**	1.000			**naive low**	**control**	.328	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.001	y			**withdrawal**	.000	y
		relief	.097				**relief**	.000	y
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	.016	y			**withdrawal**	.193	
		relief	.000	y			**relief**	.000	y
		tolerance	1.000				**tolerance**	.000	y
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.000	y
		naive low	.001	y			**naive low**	.000	y
		naive high	.016	y			**naive high**	.193	
		relief	1.000				**relief**	.314	
		tolerance	.231				**tolerance**	.086	
	relief	**control**	.047	y		**relief**	**control**	.071	
		naive low	.097				**naive low**	.000	y
		naive high	.000	y			**naive high**	.000	y
		withdrawal	1.000				**withdrawal**	.314	
		tolerance	.002	y			**tolerance**	1.000	
	tolerance	**control**	.000	y		**tolerance**	**control**	.225	
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	.000	y
		withdrawal	.231				**withdrawal**	.086	
		relief	.002	y			**relief**	1.000	

Analysis for *eat-4* - Loopyness

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	2.868	.092	
condition	5	34.658	.000	y
genotype * condition	5	4.814	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	10.597	.000	y
eat-4	**condition**	5	34.442	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	7.055	.012	y
naive low	**genotype**	1	.157	.694	
naive high	**genotype**	1	6.482	.015	y
withdrawal	**genotype**	1	.115	.736	
relief	**genotype**	1	.469	.498	
tolerance	**genotype**	1	7.603	.009	y

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	control	naive low	1.000		*eat-4*	control	naive low	.017	y
		naive high	1.000				naive high	.000	y
		withdrawal	.000	y			withdrawal	.006	y
		relief	.014	y			relief	1.000	
		tolerance	1.000				tolerance	.000	y
	naive low	control	1.000			naive low	control	.017	y
		naive high	1.000				naive high	.015	y
		withdrawal	.000	y			withdrawal	.000	y
		relief	.001	y			relief	.001	y
		tolerance	1.000				tolerance	.055	
	naive high	control	1.000			naive high	control	.000	y
		naive low	1.000				naive low	.015	y
		withdrawal	.000	y			withdrawal	.000	y
		relief	.002	y			relief	.000	y
		tolerance	1.000				tolerance	1.000	
	withdrawal	control	.000	y		withdrawal	control	.006	y
		naive low	.000	y			naive low	.000	y
		naive high	.000	y			naive high	.000	y
		relief	1.000				relief	.123	
		tolerance	.000	y			tolerance	.000	y
	relief	control	.014	y		relief	control	1.000	
		naive low	.001	y			naive low	.001	y
		naive high	.002	y			naive high	.000	y
		withdrawal	1.000				withdrawal	.123	
		tolerance	.041	y			tolerance	.000	y
	tolerance	control	1.000			tolerance	control	.000	y
		naive low	1.000				naive low	.055	
		naive high	1.000				naive high	1.000	
		withdrawal	.000	y			withdrawal	.000	y
		relief	.041	y			relief	.000	y

Analysis for *slo-1* - Speed

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	3.024	.083	
condition	5	57.374	.000	y
genotype * condition	5	5.785	.000	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	39.293	.000	y
slo-1	**condition**	5	25.054	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	.795	.378	
naive low	**genotype**	1	1.712	.199	
naive high	**genotype**	1	10.074	.003	y
withdrawal	**genotype**	1	5.625	.023	y
relief	**genotype**	1	13.836	.001	y
tolerance	**genotype**	1	.045	.833	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	.050		***slo-1***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.001	y			**withdrawal**	.000	y
		relief	.529				**relief**	.000	y
		tolerance	.000	y			**tolerance**	.000	y
	naive low	**control**	.050			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	1.000				**withdrawal**	.000	y
		relief	1.000				**relief**	.000	y
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	.000	y			**withdrawal**	1.000	
		relief	.000	y			**relief**	1.000	
		tolerance	1.000				**tolerance**	1.000	
	withdrawal	**control**	.001	y		**withdrawal**	**control**	.000	y
		naive low	1.000				**naive low**	.000	y
		naive high	.000	y			**naive high**	1.000	
		relief	.805				**relief**	1.000	
		tolerance	.000	y			**tolerance**	.025	y
	relief	**control**	.529			**relief**	**control**	.000	y
		naive low	1.000				**naive low**	.000	y
		naive high	.000	y			**naive high**	1.000	
		withdrawal	.805				**withdrawal**	1.000	
		tolerance	.000	y			**tolerance**	.008	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	1.000				**naive high**	1.000	
		withdrawal	.000	y			**withdrawal**	.025	y
		relief	.000	y			**relief**	.008	y

Analysis for *slo-1* - Efficiency

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	.471	.493	
condition	5	62.488	.000	y
genotype * condition	5	1.677	.141	

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05?
N2	**condition**	5	39.293	.000	y
slo-1	**condition**	5	25.054	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05?
control	**genotype**	1	5.565	.024	y
naive low	**genotype**	1	.870	.357	
naive high	**genotype**	1	2.712	.108	
withdrawal	**genotype**	1	.158	.694	
relief	**genotype**	1	.215	.645	
tolerance	**genotype**	1	1.516	.226	

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***slo-1***	**control**	**naive low**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.012	y			**relief**	.344	
		tolerance	.000	y			**tolerance**	.000	y
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.767				**relief**	.019	y
		tolerance	.000	y			**tolerance**	.000	y
	naive high	**control**	.000	y		**naive high**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		withdrawal	.000	y			**withdrawal**	.138	
		relief	.000	y			**relief**	.000	y
		tolerance	.002	y			**tolerance**	1.000	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	.000	y			**naive high**	.138	
		relief	.038	y			**relief**	.027	y
		tolerance	1.000				**tolerance**	1.000	
	relief	**control**	.012	y		**relief**	**control**	.344	
		naive low	.767				**naive low**	.019	y
		naive high	.000	y			**naive high**	.000	y
		withdrawal	.038	y			**withdrawal**	.027	y
		tolerance	.003	y			**tolerance**	.000	y
	tolerance	**control**	.000	y		**tolerance**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	.002	y			**naive high**	1.000	
		withdrawal	1.000				**withdrawal**	1.000	
		relief	.003	y			**relief**	.000	y

Analysis for *slo-1* - Loopyness

Two way ANOVA

Source	df	F	Sig.	p<0.05?
genotype	1	4.385	.037	y
condition	5	23.405	.000	y
genotype * condition	5	2.581	.027	y

Parallel one way ANOVAs for each genotype

genotype	Source	df	F	Sig.	p<0.05
N2	**condition**	5	12.683	.000	y
slo-1	**condition**	5	13.421	.000	y

Parallel one way ANOVAs for each condition

condition	Source	df	F	Sig.	p<0.05
control	**genotype**	1	4.394	.043	y
naive low	**genotype**	1	.069	.794	
naive high	**genotype**	1	.246	.623	
withdrawal	**genotype**	1	2.014	.164	
relief	**genotype**	1	1.881	.179	
tolerance	**genotype**	1	13.788	.001	y

Bonferroni Post-hoc tests

genotype	(I) condition	(J) condition	Sig.	p<0.05?	genotype	(I) condition	(J) condition	Sig.	p<0.05?
N2	**control**	**naive low**	1.000		***slo-1***	**control**	**naive low**	1.000	
		naive high	.086				**naive high**	.356	
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.000	y			**relief**	1.000	
		tolerance	1.000				**tolerance**	1.000	
	naive low	**control**	1.000			**naive low**	**control**	1.000	
		naive high	.189				**naive high**	.010	y
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.001	y			**relief**	.063	
		tolerance	1.000				**tolerance**	.217	
	naive high	**control**	.086			**naive high**	**control**	.356	
		naive low	.189				**naive low**	.010	y
		withdrawal	.134				**withdrawal**	.001	y
		relief	1.000				**relief**	1.000	
		tolerance	.017	y			**tolerance**	1.000	
	withdrawal	**control**	.000	y		**withdrawal**	**control**	.000	y
		naive low	.000	y			**naive low**	.000	y
		naive high	.134				**naive high**	.001	y
		relief	1.000				**relief**	.000	y
		tolerance	.000	y			**tolerance**	.000	y
	relief	**control**	.000	y		**relief**	**control**	1.000	
		naive low	.001	y			**naive low**	.063	
		naive high	1.000				**naive high**	1.000	
		withdrawal	1.000				**withdrawal**	.000	y
		tolerance	.000	y			**tolerance**	1.000	
	tolerance	**control**	1.000			**tolerance**	**control**	1.000	
		naive low	1.000				**naive low**	.217	
		naive high	.017	y			**naive high**	1.000	
		withdrawal	.000	y			**withdrawal**	.000	y
		relief	.000	y			**relief**	1.000	

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