Gene regulation in *Drosophila melanogaster in* response to an acute dose of ethanol.

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Thesis submitted for the degree of
Doctor of Philosophy.
University of Sussex, May 2010.

I hereby declare that the work contained in this thesis is entirely my own except where references and acknowledgments are made to the work of others. This thesis has never been submitted in whole or in part to this or any other University for a degree.

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Thesis submitted for the degree of Doctor of Philosophy

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Abstract

Alcohol intake causes gene expression changes resulting in cellular and molecular adaptations that could be associated with a predisposition to alcohol dependence. Expression profiling using high-throughput microarrays has recently been used to identify changes in gene expression that may be associated with alcohol dependence. To clarify the mechanisms and biology underlying alcohol dependence, bioinformatics, behavioural and genetics methodologies were employed to analyse obtained raw microarray data set that was previously generated from *Drosophila* exposed to an acute dose of ethanol.

Classical linear statistical modeling coupled with clustering and functional enrichment analyses were implemented to evaluate whole-head time series microarray data from ethanol-treated and control samples, and implicated many genes or pathways affected by acute ethanol treatment in *Drosophila* head including those involved in stress signaling, inter and intra cellular signaling, ubiquitin-mediated signaling, metabolic switches, and possible transcriptional regulatory components.

Further analysis identified interaction networks and patterns of transcriptional regulation within the set of identified genes. Seven of these genes, *ana*, *Axin*, *hiw*, *hop*, *hsp26*, *hsp83*, and *mbf1*, were verified and linked with novel roles in ethanol behavioural responses using functional tests. Additional work on two of these genes namely, *hiw* and *hsp26* also revealed a role for glia, mushroom bodies and ellipsoid body neurons as important regulators of acute ethanol response in *Drosophila*.

Finally, these studies have demonstrated that microarray analysis is an efficient method for identifying candidate genes and pathways that may be fundamental to human alcohol dependence or abuse.

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Dedication

In memory of my late mother, Chief (Mrs) F.A Awofala, 1938-2008

Table of Contents

Title

Declaration

Abstract.....i-ii

Acknowledgments……iii

Dedication……iv

Table of Contents……...v-viii

List of Figures……ix-xi

List of Tables……xii

Abbreviations……xiii-xiv

1. Introduction

1.1 Thesis Aims……2

1.2 Alcohol……2-4

1.3 Genetics of Alcohol-induced Behaviours……4-8

1.4 Molecular Mechanisms of Alcohol in the Nervous System…….8

1.4.1 Short term effects……8-11

1.4.2 Long term effects……11-12

1.5 Other signaling pathways in ethanol reinforcement……13-16

1.6 Gene transcription induced by ethanol……16-22

1.7 Ethanol metabolism……23-26

1.8 Animal models in alcohol research……26-27

1.9 *Drosophila* model in alcohol research...……...27-30

1.10 Selected Genes of Interests……30-39

1.11 Investigation Summary……...39-40

2. Experimental Materials and Methods

2.1 General fly handling and husbandry……42

2.2 Fly stocks and Genetic……..43-45

2.3 Behavioural Assays……46

2.3.1 Inebriometer Assay for Ethanol Sensitivity……..46

2.3.2 Sedation Assay for Ethanol Sensitivity……..46-47

2.3.3 Recovery Assay for Ethanol Sensitivity……47-48
2.3.4 Tolerance Assay……48
2.3.5 Heat-shock– Ethanol Cross-Tolerance Assay……49
2.3.6 Rescue Experiments……49
2.4 Ethanol Absorption Assay……49-50
2.5 Confocal Microscopy……50
2.6 Statistical Analysis…….....50-51

3. Gene Expression Analysis

3.1 Introduction……..53
3.1.1 Gene expression profiling……..53-54
3.1.2 Microarray Techniques……..54-57
3.1.3 Methods for using Affymetrix microarrays……..57-58
3.1.4 Overview of the Microarray Data Analysis Process….59
3.1.5 Data Quality……60-61
3.1.6 Normalisation……..61-64
3.1.7 Differential Expression……64-67
3.1.8 Previous analysis……..67-68
3.2 Materials and Methods
3.2.1 Microarrays Data……69
3.2.2 Data Quality……69-70
3.2.3 Normalisation……70-71
3.2.4 Differential Expression……71-74
3.2.5 Functional Clusters and Pathway Analysis……...74-76
3.2.6 Cluster Analysis……76-78
3.3 Results
3.3.1 Data Quality…….....79-80
3.3.2 Normalisation……...80-82
3.3.3 Differential Expression……83-104
3.3.4 Discussion……105-110
3.3.5 Temporal Gene Expression……111
3.3.6 Identification of Time-dependent Gene Classes in *Drosophila* Head……112-116
3.3.7 Temporal Gene Expression Discussion…….....117

3.4 Chapter Discussion…….....118-119

4. Validating Genes for Ethanol Sensitivity

4.1 Introduction……121-122

4.2 Methods……..122-123

4.3 Inebriometer……123-125

4.3.1 Inebriometer Discussion……..125-126

4.4 Sedation and Recovery Assays……..126

4.4.1 Sedation assay measures the duration of activity……..126-127

4.4.2 Dose response Discussion ……..127-128

4.4.3 Recovery assay measures the duration of intoxication.128

4.5 Ethanol sensitivity of mutant flies……129-133

4.5.1 *ana* and Ethanol Sensitivity…….....134-136

4.5.2 *Axn* and Ethanol Sensitivity……137-139

4.5.3 *hiw* and Ethanol Sensitivity……140-142

4.5.4 *hop* and Ethanol Sensitivity……..143-145

4.5.5 *hsp26* and Ethanol Sensitivity……..146-149

4.5.6 *hsp83* and Ethanol Sensitivity……..150-153

4.5.7 *mbf1* and Ethanol Sensitivity.....154-156

4.6 Chapter Discussion.....157-160

5. Testing Selected Genes for Ethanol Tolerance

5.1 Introduction……162-163

5.2 Methods……..164

5.3 Rapid tolerance can be measured with the sedation assay…….164-165

5.3.1 Kinetics of Rapid Tolerance Development and Decay.165-166

5.3.2 Flies lacking octopamine showed reduced rapid tolerance in sedation assay……..166-168

5.4 Ethanol tolerance of mutant flies……169-171

5.4.1 Mutations affecting the *hsp26* gene abolish ethanol tolerance……172-177

5.4.2 Mutations affecting the *hsp83* gene show reduced ethanol tolerance……..177-178

5.4.3 Mutations affecting the *mbf1* gene lead to reduced tolerance.....179
5.4.4 Mutations affecting the *hiw* gene show sex-specific effects on tolerance.....180
5.5 Ethanol absorption and metabolism.....181-182
5.6 Chapter Discussion.....183-187

6. Locus of Ethanol Sensitivity and Tolerance

6.1 Introduction.....189-191
6.2 Methods.....191
6.3 Results.....191
6.3.1 Neuronal expression of Hiw on ethanol sensitivity.....191-194
6.3.2 Effect of mushroom bodies in the regulation of Hiw ethanol sensitivity.....195
6.3.3 Neuronal expression of Hsp26 on ethanol sensitivity...195-197
6.3.4 Effect of mushroom bodies in the regulation of Hsp26 ethanol sensitivity.....198-199
6.3.5 Pan-neuronal expression rescue $hsp26^{EY10556}$ ethanol tolerance phenotype.....199-205
6.4 Chapter Discussion.....206-210

7. Summary and Further Work

7.1 Introduction.....212
7.2 Bioinformatics and behavioural genetics.....212-217
7.4 Possible directions for future work.....217-219

References.....221-256

Appendices

A. Supplementary data in Figures.....258-260
B. Supplementary data in Tables.....262-269

List of Figures

Chapter 1

1.1 Alcohol consumption per capital in litres of pure ethanol……4
1.2 Model of possible interaction between sHsps and ubiquitination pathway.15
1.3 Ethanol Metabolism……25
1.4 Genomic structure of *ana*^{1} allele……..32
1.5 Genomic structure of *Axn*^{EY10228} allele……..33
1.6 Genomic structure of *hiw* alleles……..34
1.7 Genomic structure of *hop* alleles……36
1.8 Genomic structure of *hsp26* alleles……37
1.9 Genomic structure of *hsp83* alleles……38
1.10 Genomic structure of *mbf1*^{2} allele……39

Chapter 3

3.1 The central processes of gene expression: transcription and translation…..54
3.2 Probesets are made up of multiple probes used interrogating the sequence of a particular gene……57
3.3 Perfect Match and Mismatch Probes……...57
3.4 The procedure of an Affymetrix microarray experiment……58
3.5 The whole microarray data analysis workflow used in this thesis……59
3.6 Pseudo-images of two of the *Drosophila* chips used……61
3.7 Volcano Plot with moderated t-statistics made from the 2 h array data…...65
3.8 R code for the 2 h (T2) differential gene expression analysis showing the design and contrast matrix defined within the LIMMA statistical package……...72
3.9 R code for the time series (TS) differential gene expression analysis showing the design and contrast matrix defined within the LIMMA statistical package……...72
3.10 Boxplots of nine arrays probe-level 2h array data……...80
3.11 NUSE and RLE plots for the 2 h array data……80
3.12 Boxplots of nine arrays normalised expression-level 2 h array data……..81
3.13 Boxplots of the time series data before and after normalisation……82
3.14 Correlation of fold change between RMA and GCRMA algorithms……...84
3.15 Global pathway analysis for up-and down-regulated gene datasets……103

3.16 A network model of common up- or down-regulated genes……104
3.17 Clustering of time-dependent changes in acute ethanol response……116

Chapter 4

4.1 Ethanol sensitivity of the *Drosophila* isogenic wild-type strains……125
4.2 Effects of ethanol dose on male wild-type 2447 (Ctl) flies……127
4.3 Recovery from intoxication of normal ethanol-sensitivity of 2447 wild-type flies……128
4.4 Ethanol sensitivity of the candidate genes……133
4.5 *ana*^{1} flies have reduced ethanol sensitivity……..136
4.6 *Axn*^{EY10228}/+ flies have reduced ethanol sensitivity……..139
4.7 *hiw* flies have increased ethanol sensitivity……..142
4.8 *hop* flies have reduced ethanol sensitivity……..145
4.9 *hsp26* flies have normal ethanol sensitivity only in sedation assay……..149
4.10 *hsp83* flies have reduced ethanol sensitivity……...153
4.11 *mbf1*^{2} flies have increased ethanol sensitivity……156

Chapter 5

5.1 Ethanol tolerance measured in the sedation assay……165
5.2 Kinetics of Tolerance acquisition and decay……166
5.3 Role for octopamine in rapid tolerance in sedation assay……168
5.4 Rapid ethanol tolerance of all the mutants tested ……171
5.5 Effect of *hsp26* in ethanol tolerance……173
5.6 Kinetics of tolerance behaviour in *hsp26* mutant flies……..174
5.7 *hsp26* ethanol tolerance after heat shock……176
5.8 Tolerance is restored in *hsp26* and *Tbh* double mutant flies……177
5.9 Effect of *hsp83* in ethanol tolerance……178
5.10 Effect of *mbf1*^{2} in ethanol tolerance……179
5.11 Effect of *hiw* in ethanol tolerance……...180
5.12 Ethanol concentrations in flies after their ethanol treatment……...182

Chapter 6

6.1 Transgenic expression of Hiw in specific brain regions alters ethanol sensitivity in the sedation and recovery assays……194

6.2 Overexpression of Hsp26 in the whole neurons (elav (458)) and mushroom bodies (OK107 and 201Y) led to significant alterations in ethanol sensitivity but RNAi expression of *hsp26* in these structures showed no significant alterations in ethanol sensitivity……..197
6.3 $Hsp26^{RNAi}$ expression in the nervous system reduced tolerance……..200
6.4 Hsp26 expression in the nervous system reduced tolerance……201
6.5 Overexpression of Hsp26 in the whole neurons (elav (458)) and mushroom bodies (OK107 and 201Y) led to significant alterations in ethanol sensitivity but no significant effect on tolerance……..202
6.6 Spatially restricted functional knockdown of Hsp26 in the brain with repo (7415) led to reduced ethanol tolerance……204
6.7 Hsp26 expression in the MBs did not rescue rapid tolerance……..205
6.8 Expression of P[GAL4] lines……..205
A.1 Boxplots showing GCRMA normalised pooled time course array data…258
A.2 Histograms of the 2 h microarray data……..258
A.3 MA plots of the 2 h microarray data……..259
A.4 Hierarchical cluster of the 2 h microarray data……259
A.5 Genetic crosses used to create P[GAL4] lines 201Y and Elav and the P[UAS-hsp26] in $hsp26^{EY10556}$ mutant background……260

List of Tables

Chapter 1

1.1 Gene expression profiling on human and animal models used in alcohol studies……18-22

Chapter 2

2.1 Fly stocks with their annotations……...44-45

Chapter 3

3.1 A summarised information on all the *Drosophila* chips used……70

3.2 Comparison between RMA and GCRMA 101 upregulated gene overlaps……86-93

3.3 Comparison between RMA and GCRMA 54 downregulated gene overlaps……94-97

3.4 The first three significance (*p* value) DAVID functional clusters for up-and down-regulated genes……100

3.5 Pearson correlation coefficient similarity matrix on the time-dependent gene expression estimates……111

Chapter 4

4.1 Ethanol Sensitivity of Candidate Genes……131-132

Chapter 5

5.1 Rapid ethanol tolerance of the four genes tested……..170

Chapter 7

7.1 Some identified genes implicated in ethanol response……..213

Appendix.B

B.1 Functional clusters of ethanol sensitive genes for up-regulated genes.262-264

B.2 Functional clusters of ethanol sensitive genes for down-regulated genes…… .265-266

B.3 Genes common in three microarray studies of EtOH regulation in *Drosophila*……267-269

Abbreviations.

°C	Degrees centrigrade
µl	Microlitre
ADH	Alcohol Dehydrogenase
ALDH	Aldehyde Dehydrogenase
ANOVA	Analysis of Variance
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatase
BAC	Blood Alcohol Concentration/Content
bp	Base Pairs
CNS	Central Nervous System
CO_2	Carbon Dioxide
DA	Dopamine
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DNA	Deoxyribonucleic acid
EMS	Ethyl methanesulfonate
ENU	*N*-ethyl-*N*-nitrosourea
FC	Fold Change
FDR	False Discovery Rate
EtOH	Ethanol/ alcohol
g	gram
GABA	γ-aminobutyric acid
GCRMA	Guanine-Cytosine Robust Multichip Average
GFP	Green Fluorescent Protein
GO	Gene Ontology
h	hour
H_2O	Water
HPLC	High Performance Liquid Chromatography
kb	Kilobase pairs
l	litre
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LIMMA	Linear Modeling for Microarray Analysis

MEOS	Microsomal Ethanol Oxidising System
MET	Mean Elution Time
min	Minute
ml	Millilitre
mM	millimolar
mRNA	messenger RNA
MRT	Mean Recovery Time
MST	Mean Sedation Time
NAC	Nucleus Accumbens
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced form of Nicotinamide Adenine Dinucleotide
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NIH	National Institute of Health
nm	nanometre
NMDA	*N*-methyl-D-aspartate
NUSE	Normalised Unscaled Standard Error
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
RLE	Relative Log Expression
RMA	Robust Multichip Average
RNA	Ribonucleic acid
SD	Standard Deviation
SE	Standard Error
SNK	Student Newman Keuls
STEM	Short Time Series Expression Miner
TCA	Tricarboxylic Acid
UAS	Upstream Activating Sequence
UPS	Ubiquitin Proteasome System
VTA	Ventral Tegmental Area
WHO	World Health Organisation
w/v	Weight/Volume

Chapter 1.

Introduction.

1 Introduction

1.1 Thesis Aims

- Compare and trace the gene expression profiles obtained from *Drosophila* head exposed to an acute dose of ethanol over a number of different time points.
- Identify genes, pathways and networks that are up- or down-regulated in *Drosophila* head following acute ethanol exposure.
- Validate some of the identified genes for ethanol sensitivity using behavioural genetics approaches.
- Determine what effect(s) mutations have on rapid ethanol tolerance of some of the validated genes.
- From the mutant analysis, select genes to use in characterising *Drosophila* brain loci involved in ethanol response.
- Discuss the link between ethanol, identified genes and pathways (including specific genes of interest), and behaviour in order to aid future direction of ethanol research in both *Drosophila* and mammalian models.

1.2 Alcohol

Alcohol has been consumed for ages and with special roles in medicine and religious rituals of many cultures and societies. For instance, during the early periods of alcoholic drinks, many claims have been brought forward that alcohol can be used to treat a variety of illnesses such as treating of snake bites, gout and veneral infections and influenza (Anderson and Baumberg, 2006).

A growing body of evidence indicates that low to moderate alcohol consumption is associated with lowered risk of diabetes and cardiovascular diseases (Health Risks and Benefits of Alcohol Consumption, 2000; Mukamal and Rimm, 2008). Moderate alcohol use has also been linked to a reduction in stroke, osteoporosis and Alzheimer's diseases among others (Spanagel, 2009). This reducing effect may be related to the ability of alcohol to increase plasma high density lipo-protein-cholesterol levels (Sesso, 2001). In addition, a substantial proportion of the benefit of moderate drinking is due to the pure ethanol component of alcoholic beverages

such as resveratrol, a polyphenol found in red wine, that can increase the function of endogenous antioxidants system (Spanagel, 2009).

Conversely, excessive drinking can lead to neuronal loss and a variety of pathological lesions including breast, colorectal and prostrate cancer (Mukamal and Rimm, 2008). Other heath risks of heavy drinking include cirrhosis of the liver, stroke, high blood pressure and mental disorders of various types **(**Health Risks and Benefits of Alcohol Consumption, 2000; Mukamal and Rimm, 2008). Thus while low to moderate alcohol use may be beneficial, people do not usually drink on health grounds. The most commonly reported reasons for drinking include stress reduction, mood elevation, increased sociability and relaxation (Health Risks and Benefits of Alcohol Consumption, 2000) and these often lead to abuse which driving force in most cases is the development of an addictive behaviour (Spanagel, 2009).

Alcohol abuse has a high comorbidity with other psychiatric disorders with people who suffer anxiety disorders and depression regularly make use of alcohol as a kind of self-medication (Spanagel, 2009). Chronic use of alcohol in this way can contribute to altered immune regulation leading to immunodeficiency resulting in increased susceptibility to bacteria pneumonia, tuberculosis, and other infectious diseases (Cook, 1998).

Today, the great majority of countries regularly consume alcohol with ~100 billion Euros spent on alcoholic beverages only in the European countries (Spanagel, 2009). This is reflected by the high rate of alcohol consumption per capital in litres of pure ethanol per year (Figure 1.1). Throughout the world, Luxemburg has the highest level of consumption with more than 13 litres per year; the alcohol consumption in north America in the last decade average 4.5 litres per year while Nigeria has the highest alcohol consumption in Africa with average 10.5 litres per year (Figure 1.1).

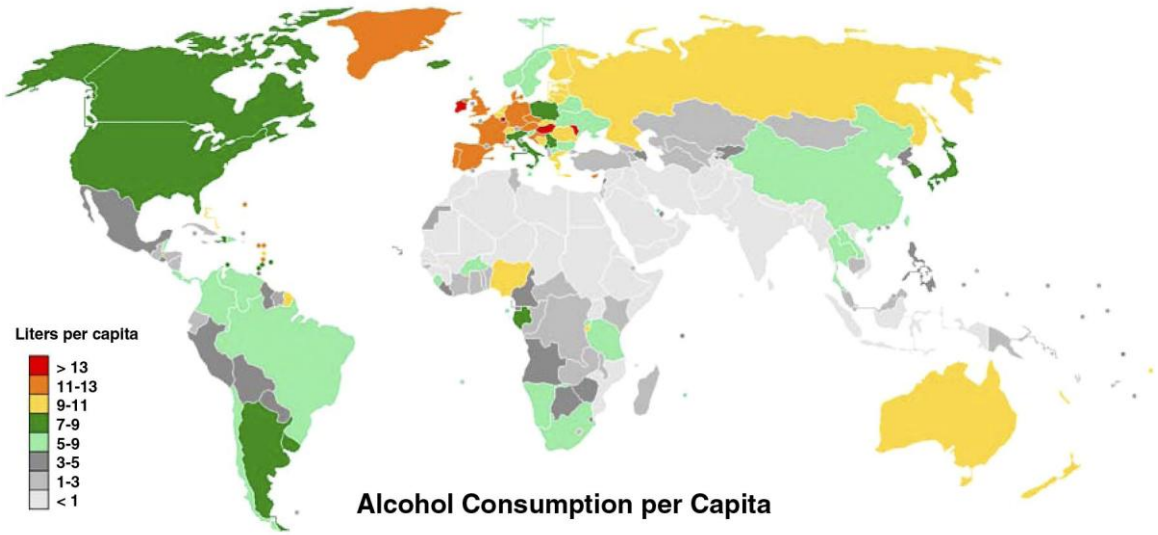


Figure 1.1: Alcohol consumption per capital in litres of pure ethanol.
Source: Spanagel, R (2009). Alcoholism: A Systems Approach from Molecular Physiology to Addictive Behaviour**.**

Notwithstanding the immense positive contribution of alcoholic beverages production and sale to the economy of these countries in terms of revenue and employment, alcohol use and abuse affects all social and ethnic groups (Spanagel, 2009). For example, in nearly every family in the Western societies, there will be someone who has directly or indirectly suffered from the alcohol-attributable disease, injury and violence that now persistently drain the health, welfare, employment and criminal justice sectors (Anderson and Baumberg, 2006).

1.3 Genetics of Alcohol-induced Behaviours

It is no longer new that both genetic and environmental factors influence the risk of alcohol dependence. Indeed, alcohol dependence is the result of cumulative responses to alcohol exposure, the genetic make-up of an individual and the environmental perturbations over time (Spanagel, 2009). Some of the evidence of genetic influence of alcoholism elucidated by previous studies includes:

Twin, Family and Adoption Studies: The classic twin study designs have shown that genetic factors are important in determining susceptibility to alcohol dependence (Ducci and Goldman, 2008). The design compares the similarities for a condition of interest between monozygotic (MZ, identical) twins and dizygotic (DZ, fraternal) twins, in order to evaluate the extent of genetic influences, or heritability of the condition. The determination of heritability from these studies is based on the fact that the MZ twins are genetically identical, whereas, DZ twins share only half of their genes. Thus if genetic effects were present then MZ twins should be more alike than DZ twins thereby allowing for estimation of such genetic contribution. For example, in a study of 1,030 U.S. Caucasian female twin pairs, concordance of alcoholism was found to be consistently higher in MZ than DZ twin pairs, and the heritability of alcoholism in women found to be between 0.50 and 0.60 (reviewed in Enoch and Godman, 2002). This study showed that alcohol disorders are not totally influenced by genotype but there is also the involvement of environmental interactions.

Though the results of twin studies were not geared towards identifying specific genes influencing alcoholism, yet they provide vital information on the disease's genetic impact, which aspect of it are most heritable, whether the same genes are influencing disease in both genders, and whether multiple diseases share any common genetic influences (Anderson et al., 2005). When the data on twins are augmented by the data on their family members, the study is termed a twin/family study. The twin/family studies provide more informed and precise data about whether parents transmit a behavioural condition to their offspring genetically or via some aspect of familial environment (cultural transmission) (Anderson et al., 2005).

Adoption studies have shown that alcoholism in biological parents predicts alcoholism in children even when the child is reared by unrelated adoptive parents (Sher, 1997). Studies have found reductions in alcoholism occurrence in subsequent generation after removal from home with alcoholic fathers (Cloniger et al., 1981), increased risk of alcoholism in adopted out individuals whose biological background included alcoholism but no increased risk if only the adoptive parents exhibited the trait (Cadoret and Gath 1978).

Linkage Studies: Linkage studies have also been undertaken to identity candidate chromosomal regions susceptible to alcoholism. In a study carried out on large sample families in the US by the Collaborative Study of the Genetics of Alcoholism (COGA) regions on DNA affording susceptibility to alcohol dependence were highlighted. Regions on chromosomes 1, 2, 3, 7 and 8 were reported (Foroud et al., 2000; Edenberg, 2002). The strongest evidence was for regions on chromosome 1 and 7, with more modest evidence for a region on chromosome 2. This evidence was provided from the initial analysis of 987 people from 105 families in the sample of COGA. Another sample (latter sample) called the replication sample comprising 1,295 people from 157 families, confirmed the former findings though with less statistical support (Edenberg, 2002).

Comparing and contrasting these two independent samples have shown that genes increasing the risk of alcoholism were located in the same regions of chromosome 1 and 7 (Foroud et al., 2000). Some notable candidate genes in the chromosome 1 region include adenosine 3':5'-cyclic monophosphate-specific, phosphodiesterase 4B, gamma-5 guanine nucleotide-binding protein, and protein kinase C-like 2 and those encoding ion channels (e.g. calcium-activated chloride channel and voltage-gated potassium channel). Candidate genes in chromosome 7 region include neuropeptide Y (a *Drosophila* homolog of *neuropeptide F* recently implicated in ethanol sensitivity (Wen et al., 2005)), adenylate cyclase , syntaxin 1A and other receptors such as the glutamate (GRM3) and cholinergic receptor (CHRM2) (Foroud et al., 2000). The replication sample however showed no additional evidence for alcoholism susceptibility on chromosome 2 region (Edenberg, 2002). Conversely, the strongest evidence in the replication sample for a region containing genes affecting the risk for alcoholism was on chromosome 3, which had shown no evidence of being linked with alcoholism in the initial sample (Edenberg, 2002). However, when both the initial and replication samples were combined the region on chromosome 1 provided the strongest evidence for a susceptibility gene in the combined sample. In addition, this new evaluation detected a region on chromosome 8 that was linked with the risk for alcoholism (Foroud et al., 2000; Edenberg, 2002). Finally and interestingly, when the analysis of non-alcoholic sibling pairs in the initial sample was carried out, it produced evidence for a

protective region on chromosome 4, in the general vicinity of the alcohol dehydrogenase (ADH) genes (Edenberg, 2002).

Association studies: unlike linkage analyses which are used to identify a broad chromosomal region that may likely contain a gene contributing to behavioural disorder, association studies (Dick and Foroud, 2002) are able to accurately link gene or genes contributing to a disorder or phenotype of interest. This usually involves the use of candidate gene analysis to test the association between a particular allele of the candidate gene and a specific behaviour. Such a candidate gene is chosen either based on its suspected role in the behaviour or other known information relating to the behavioural outcome. In addition, the gene could be chosen because it lies in a DNA region known to affect or linked to the disorder through linkage studies. For instance, in *Drosophila*, a mutant called *cheapdate* exhibited increased sensitivity to ethanol (Moore et al., 1998). This mutant is an allele of a gene named *amnesiac* important in cAMP signaling pathways and as a result genes involved in this pathway e.g. *rutabaga* and *DCO* have been reasonably chosen as candidate genes influencing ethanol sensitivity (see section 1.5).

The candidate gene approach is often used in quantitative trait loci (QTL) which defines DNA regions that may contain one or more genes related to the development of a certain quantitative trait (Goate and Edenberg, 1998). Recently, QTL analyses are combined with behavioural expression microarray analysis. Such behavioural and expression QTLs (bQTL and eQTL) are very useful in identifying the most promising candidate genes among the plethora of genes identified during the initial microarray screening. This is because they help to describe areas in the genome that control the phenotype of interest and the regulatory elements in the genome that control the mRNA transcription level of the candidate genes (Saba et al., 2008). It should be noted that any association study (be it family- or population-based association studies, reviewed in Dick and Foroud, 2002) tests the null hypothesis that the frequency of a particular trait is the same in both patients and controls. Such studies have suffered from lack of power due to small subject numbers, poor selection of control subjects, and over-emphasis on markers with

low probability of involvement (see Buckland, 2001 for a detailed review of problems associated with association studies).

1.4 Molecular Mechanisms of Alcohol in the Nervous System

Ethanol was once thought to exert it effects on the cells by altering the physical properties of lipid bilayer membranes of the cells, however, recent studies have overwhelmingly shown that ethanol interacts with and modify the functions of proteins of the nervous system including ion channels and second messenger proteins (Peoples et al., 1996; Gordis, 1998; Harris, 1999). Alcohol's effects on the brain and behaviour depend on an individual's blood alcohol concentration (BAC) with low doses having stimulating effects and higher doses depressant effects. Individuals differ in their sensitivities and tolerance to acute ethanol intoxication under the same conditions as a result of their varied responses due to differences in their metabolic, physiological, cognitive or motor functions (Oscar-Berman and Marinkovic, 2007). Though a series of studies on the depressive effects of ethanol in the nervous system have been carried out, the molecular mechanisms of how this drug exerts its effects on the nervous system are still under investigation.

For years alcohol researchers have worked to define the cellular and molecular mechanisms by which alcohol produces its short and long-term effects such as intoxication, dependence and withdrawal (Gordis, 1998). A brief description of these two broad categories (effects) with some of the major changes associated with each of them is presented here:

1.4.1 Short term effect

This effect is characterised by the intoxicating behaviour and defines the immediate effects of ethanol on the nervous system. This involves initial euphoria, increased activity and relief from anxiety and social inhibitions at low doses. Progressive increase in dose level is attributed to a decline in physical activity, uncoordination, reduced response to sensory stimulation and disproportionate impairment of executive functions such as planning and working memory (Oscar-Berman and Marinkovic, 2007). Finally, at higher doses, effects such as drowsiness, hypnosis, anaesthesia and death due to respiratory failure occur (Julien, 2004). Acute effects

of ethanol have two major opposing functions on gene expression e.g. inhibitory and excitatory functions involving the potentiating and inhibiting of ion channels respectively.

(a) Pathways inhibited by acute ethanol

Ethanol inhibits ion channels that have excitatory functions. Some of these ion channels with excitatory functions are:

NMDA: The major excitatory (i.e. it affects neurons in a way that increases their activity) neurotransmitter in the CNS is glutamate with more than half of the synapses in the brain which are excitatory making use of glutamate as their neurotransmitter (Harris 1999, Wirkner et al., 1999). The N-methyl-D-aspartate (NMDA) receptor is a ionotrophic (i.e. ligand-gated ion channels allowing cation influx) receptor types of glutamate containing several subunits with different functions (Spanagel, 2009). NMDA receptors are the main targets for research as they are involved in learning and memory, long term plasticity and depression (Diamond and Gordon, 1997) and are permeable to calcium and blocked by magnesium at resting potentials (Wirkner et al., 1999). Acute exposure to ethanol antagonise NMDA receptors, inhibits calcium flux into cells thereby inhibiting the excitatory effect of glutamate-activated NMDA receptor function (Wirkner et al., 1999).

Voltage-gated calcium channels: Voltage-dependent calcium channels consist of several subunit complexes defined by variations in their electrophysiological and pharmacological features (Diamond and Gordon, 1997). These non-ligand ion channels also constitute a primary target of ethanol. One of these channels known as L type, found mainly in the cell bodies and proximal dendrites in many tissues including the brain, heart, smooth muscles and pancreas, has particularly been implicated in the acute ethanol action (Walter and Messing, 1999). Other channels including N-, T- and P-type channels have also been similarly implicated in the acute effects of ethanol (Diamond and Gordon, 1997; Walter and Messing, 1999). These channels are activated through depolarization stimulated calcium influx into neurons thereby increasing their excitability. Acute exposure to ethanol has been

shown to inhibit these channels due to decrease in open probability, primarily by shortening the open duration of the channels (Walter and Messing, 1999).

(b) Pathways potentiated by acute ethanol

Ethanol potentiates ion channels that have inhibitory functions. Some of these ion channels with inhibitory functions are:

GABA: The γ-aminobutyric acid (GABA) inhibitory (i.e. it affects neurons in a way that reduces their activity) neurotransmission system has been suggested, like most other depressant such as benzodiazepines and barbiturates, to offer useful insights into some of the depressant effects of ethanol action on the nervous system (Honse et al., 2004). Several lines of evidence indicates that GABA is involved in many of the behavioural effects of ethanol such as motor incoordination, anxiety reduction, sedation, withdrawal signs and preference for alcohol (Kumar et al., 2009). For instance, in *Drosophila*, *$GABA_B$ receptor 1* (*$GABA_B$ R-1*) has been implicated in behaviour-imparing effects of ethanol as shown from RNAi and pharmacological evidence (Dzitoyeva et al., 2003). In mammals, another GABA receptor type, $GABA_A$ receptor (an ionotrophic ligand-gated receptor) is composed of several subunits with evidence showing differences in their sensitivity to ethanol (McCool et al., 2003). Acute ethanol has been shown to potentiate the activity of these receptors in rats, increasing the calcium flux through the ligand-gated ion channel and increasing GABAergic inhibition (Mhatre and Ticku, 1992). In mice, ethanol mediates the binding of GABA to $GABA_A$ receptor leading to increase in activity and therefore inhibition (Harris and Mihic, 2004). $GABA_A$ receptors are found at high level in the cerebral cortex, hippocampus and cerebellum (Julien, 2004).

Glycine: The strychnine-sensitive glycine receptors (GlyR) constitute the major inhibitory receptor in the brain stem and spinal cord and are found in other brain locations such as the cerebellum, thalamus, hypothalamus and cerebellar cortex (Mihic, 1999). Similar to the structure of $GABA_A$ receptors, the GlyR is composed of five subunits surrounding an ion conducting pore (Mohammadi et al., 2005). Ethanol has direct effects on glycine receptors (Spanagel, 2009). The subunit composition of GlyR is critical in the response to ethanol with the acute ethanol at

intoxicating concentrations shown to potentiate GlyR (like $GABA_A$) in many preparations (Suzdak et al., 1986; Celentano et al., 1988; Mihic, 1999 and Eggers and Berger, 2004).

Acetylcholine: The nicotinic acetylcholine receptors (nAChR) is a ligand-gated ion channels with excitatory effects in the human CNS (Dick and Agrawai, 2008). Acetylcholine appears to be involved in such processes as arousal, reward, learning and short time memory (Dick and Agrawai, 2008).The nAChR has been found to be primarily expressed in the cerebral cortex and some limbic regions with ethanol sensitivities affecting some brain regions more than the others (Spanagel et al., 2009). The nAChR appears to be involved in the acute locomotor response to ethanol in both humans and animal models (Kamens and Phillips, 2008). Ethanol has been reported to potentiate neuronal nAChR (Narahashi et al., 1999).

Serotonin: Serotonin is made by small discrete clusters of neurons located at the base of the brain with the serotonergic neurons connecting to other neurons located throughout the CNS including neurons in the cerebral cortex and other forebrain structures (Lovinger, 2008). Serotonin thus has the capacity to influence a variety of brain functions including sensations related to environmental stimuli, pain perception, learning and memory, and sleep and mood (Lovinger, 2008). Studies have shown evidence of direct link between alcohol intake and condition of serotonin neurotransmission in man (LeMarquand et al., 1994). Ethanol has been shown to potentiate these receptors by increasing their open probability with the receptors reported to have been found in many areas of the peripheral and CNS including inhibitory interneuron in the fore brain, cerebral cortex and hippocampal gyri (Lovinger, 1999).

1.4.2 Long term effect

This defined adaptive effects of ethanol on the nervous system. Example includes adaptive changes that develop in alcoholics because of prolonged drinking (Diamond and Gordon, 1997). It occurs when ethanol is consumed chronically, thereby making the nervous system adapt to the presence of the drug. The neuroadaptations produce changes in sensitivity to ethanol's effects following

repeated exposure (e.g. sensitisation and tolerance) and a withdrawal state following discontinuation of alcohol use (Gilpin and Koob, 2008). Chronic effects of ethanol have been found to affect many genes mostly in a manner opposite to the acute effects of ethanol. Only few of these are briefly discussed below:

NMDA: One of the adaptive responses in the nervous system to chronic ethanol exposure is an upregulation of NMDA receptors (Diamond and Gordon, 1997). In rats, chronic ethanol causes an increase in NMDA receptors (Sanna et al., 1993). Similarly, upregulation of NMDA receptors have also been found in human alcoholics (Michaelis et al., 1990). This increase in NMDA receptor is likely a compensatory change induced by the inhibitory effect of acute alcohol (Fadda and Rossetti, 1998). This ethanol-induced upregulation of this channel has been shown to cause overactivity of the nervous system that could result in excitotoxic neuronal cell damage in several neurological disorders including strokes and seizures (Rothman and Olney, 1995) while inhibition of NMDA receptors can attenuate this excitotoxic neuronal cell damage (Chandler et al., 1993).

GABA: Chronic ethanol exposure alters the GABA systems (Gilpin and Koob, 2008). One of these alterations involves changes in the subunit composition that make up the $GABA_A$ receptors in certain brain regions (Lovinger, 2008; Gilpin and Koob, 2008). For instance, chronic ethanol causes a decrease in the $GABA_A$ receptors α1 peptides in amygdala and α4 subunit in the amygdala and nucleus accumbens of mesocorticolimbic regions of rat brain (Papandeas et al., 2001). The main effect of this chronic alcohol effects is to make the brain hyperexcitable during withdrawal from chronic alcohol exposure (Lovinger, 2008).

Serotonin: Repeated alcohol consumption has recently been shown to downregulate serotonin functions within the nucleus accumbens in mice by reducing the extracellular concentration of serotonin (Ward et al., 2009).

1.5 Other signaling pathways in ethanol reinforcement

Ethanol is known to affects the activity of multiple signal transduction systems. Some of these include:

cAMP signal transduction: Ethanol has been reported to affect receptor-mediated cAMP signal transduction in many biological preparations, and vary with the expression of certain types of adenylyl cyclases (reviewed in Diamond and Gordon, 1997). cAMP and cAMP-dependent protein kinase A (PKA) pathway is a signaling system induced by exposure to ethanol and with the expression of numerous ethanol-responsive genes regulated by PKA (Spanagel, 2009). Acute exposure to ethanol has been shown to potentiate receptor-activated cAMP production (Asyyed et al., 2006, Diamond and Gordon, 1997). In contrast, chronic exposure to ethanol causes a decrease in receptor-stimulated cAMP production in many preparations (Boyadjieva and Sarkar, 2006, Gobejishvili et al., 2006). The importance of cAMP-PKA signaling in ethanol response has been demonstrated in mice (Wand et al., 2001) and in flies (Moore et al., 1998). Mice lacking one Gsα allele and mice with reduced neuronal PKA activity (lower AC activity) in NAC have a decreased alcohol consumption compared with their wild-type littermates. Further investigation shows that genetic reduction of cAMP-PKA signaling makes mice more sensitive to the sedative effects of ethanol while transgenic expression of a constitutively active form of Gsα leading to increase AC activity in neurons within the forebrain, results in decreased sensitivity to the sedative effects of ethanol (Wand et al., 2001). In *Drosophila*, *cheapdate* allele of the *amnesiac* gene with increased ethanol sensitivity encodes a neuropeptide believed to activate the cAMP pathway by action on adenylate cyclase. Further investigation showed that other mutants namely *rutabaga* and *DCO* in the same cAMP pathway also display increased sensitivity to acute ethanol exposure when cAMP levels were lower than normal (Moore et al., 1998). While the cAMP-PKA signaling system mediates the effects of ethanol, it also influences the cAMP response element binding protein (CREB)-mediated processes. For instance, CREB activity has been shown to be upregulated in response to ethanol-induced increase in cAMP via PKA activity (Chao and Nestler, 2004). Thus, altered CREB functions affects its regulatory role on many ethanol-responsive genes including neuropeptides, neurotransmitter

synthesizing enzymes, neurotransmitter receptors, signaling proteins, and other transcription factors (Chao and Nestler, 2004).

Stress pathways: Recent research has brought about a hypothesis that transition to alcohol dependence involves the dysregulation of not only the neural circuits involved in rewards but also the circuits that mediates behavioural responses to stressors (Gilpin and Koob, 2008). The role of stress pathway(s) in the response to ethanol has previously been suggested (Scholz et al., 2000; Scholz et al., 2005; Piper et al, 1994; Alexandre et al., 2001) and with demonstrated overlaps in some changes induced by ethanol and heat stress (Piper et al., 1994; Scholz et al., 2005). In *Drosophila*, a transcription factor, *hangover* encoding a zinc finger protein has been shown to be deficient in both ethanol tolerance and heat-ethanol cross-tolerance, indicating that cellular changes induced by ethanol and heat overlap (Scholz et al., 2005). This also suggests that stress response may mediate the development of ethanol tolerance. Similarly, many genes including heat shock proteins, and transcription factors have been shown to be induced following heat shock and ethanol treatments in yeast (Piper et al., 1994). Changes in catechol-amine synthesis and activity have been shown in response to ethanol and other stressors (Hirashima et al., 2000). Indeed ethanol causes changes in the activity of many genes including transcription factors and chaperones and this may be through interaction with many targets including ion channels, transporters, neurotransmitter receptors and enzymes that produce second messengers (Diamond and Gordon, 1997). Other stress-related systems that may be important in the development of alcohol dependence include the signaling molecule corticotrophin-releasing factor (CRF), neuropeptide-Y (NPY), vasopressin and neurokinin (reviewed, in Gilpin and Koob, 2008). Notably, complete knock-out of neurokinin-1 receptors by genetic methodologies suppresses alcohol drinking in mice while antagonism of these receptors reduces craving and neuroendocrine responses to alcohol-related cues and negative affective images in human alcoholics (Gilpin and Koob, 2008).

Ubiquitination pathways: The ubiquitin-mediated proteolysis by the proteasome has a crucial biological role as it regulates the levels of a large number of key proteins that participate in cellular regulation, inflammation, and in some cases etiology of

disease (Donohue, 2002). The ubiquitin-conjugating systems are key players in the tagging of proteins with ubiquitin usually marking them for degradation by the 26S proteasome (Joanisse, et al., 1998). In man, ethanol consumption affects the levels of ubiquitin: serum concentrations of both free ubiquitin and multi-ubiquitin chains are reportedly higher in patients with alcoholic cirrhosis than in normal subjects and those with hepatic alcoholic fibrosis and viral liver cirrhosis (Donohue, 2002). The significance of ubiquitination pathway in alcohol dependence is further suggested by the role of ubiquitin-proteasome system (UPS) in synaptic plasticity. Indeed, synaptic plasticity is regulated by two opposing processes involving regulated protein synthesis and the selective protein degradation mediated by the UPS (Ossipov et al., 2007). Importantly, neuronal activity regulates synaptic composition through the UPS, and thus provides a link between synaptic activity, protein turnover, and the functional reorganization of synapses (Ossipov et al., 2007).

The UPS also function in cooperation with the stress pathway to regulate interactions between destabilised proteins and prevent their precipitation under ethanol stress (Treweek et al., 2000). Fig. 1.2 shows the relationships between small heat shock proteins (sHsps) and ubiquitin conjugating enzyme 9 (UBC9) in flies.

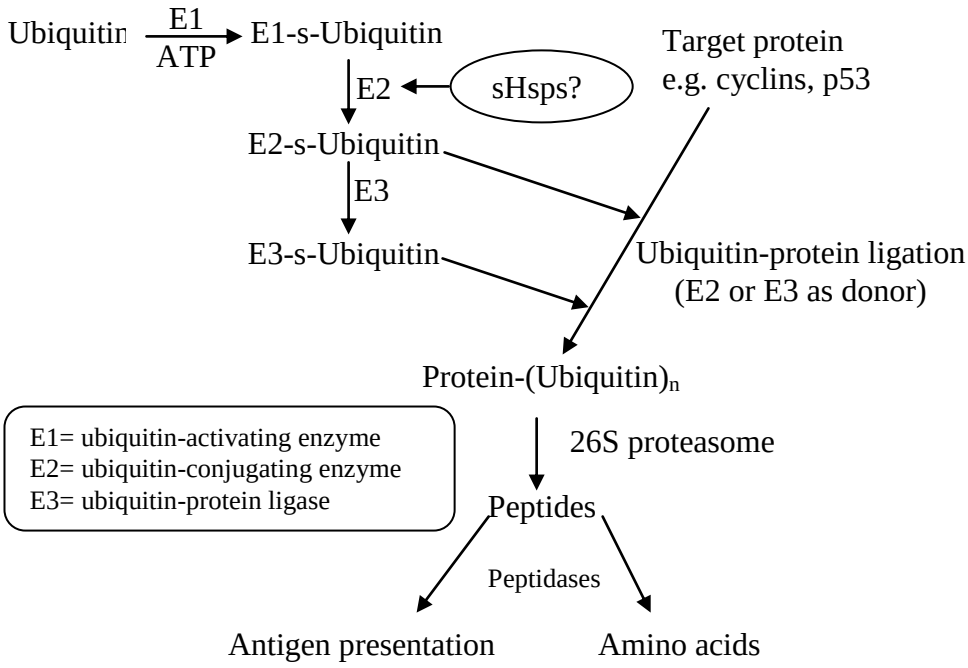


Figure 1.2. Model of possible interaction between sHsps and ubiquitination pathway. Redrawn from Joanisse et al., 1998.

As shown in the model (Fig. 1.2) the ubiquitin conjugating enzymes are key players in the tagging of proteins with ubiquitin, usually marking them for degradation by the 26S proteasome. It appears that some substrates are proteolysed only with the help of chaperones, suggesting that 'chaperone presentation' to the proteasome might be a feature of protein degradation. Indeed, evidence has linked chaperones with proper proteasome function (Glickman and Ciechanover, 2002). UBC9 from other species is known to be involved in the degradation of a number key cellular proteins, including cyclins, and IkB, the inhibitor of transcriptional regulator NF*k*B activation (Joanisse et al., 1998).

The components of the UPS are found in all tissues and the pathway is essential for cell viability (Donohue, 2002). Upon alcohol administration these tissues are subjected to oxidative stress causing a rapid response by the UPS, as indicated by the formation of ubiquitylated proteins and their subsequent hydrolysis (Donohue, 2002). Expression profiling studies in prefrontal cortex have identified differentially expressed genes involved in many processes including protein trafficking and ubiquitination (Flatscher-Bader et al., 2006). Indeed, ethanol administration impairs ubiquitin-mediated proteolysis either by partially inactivating the proteasome probably due to oxidative modifications of the enzymes or by blocking ubiquitylation of protein substrates by aldehydes and other reactive species (Donohue, 2002).

1.6 Gene transcription induced by ethanol

Acute or chronic effects of ethanol are known to be mediated by CREB whose target genes including those that control neurotransmission, cell structure, signal transduction, transcription, and metabolism are involved in mediating behavioural responses to ethanol (Spanagel, 2009). However, given that alcohol abuse is a complex disorder associated with diverse changes in the brain that affect behaviour, many other ethanol-responsive genes that are CREB-independent may be implicated. It thus follows that a better understanding of this complex trait requires identification of novel ethanol-responsive target genes and their products. This could be made possible by gene expression profiling approaches.

The study of gene expression profiling of cells and tissues is a major tool for discovery in biology. This is because microarrays allow description of genome-wide transcriptional changes in two distinct biological states of normal and abnormal cells or tissues. The results of such experiments are expected to offer new insights into the biology governing the physiological responses to ethanol. In addition, an unbiased and systematic study of gene expression profiling should allow the discovery of a functional relationship between genes. Such work has the potential to establish novel genes and pathways of disease for alcohol disorder and or dependence.

Using microarrays, researchers are able to study genetic changes that occur together to affect a given phenotype. Expression profiling with DNA microarrays has been used to identify changes in brain gene expression that are relevant in dissecting pathways mediating complex behavioural traits (Mirnics et al., 2000). In addition, researchers have used a variety of sophisticated approaches including microarrays to identify a variety of genes that are differentially expressed by alcohol in human and animal models (Worst and Vrana, 2005, Flatcher-Bader et al. 2006, Kwon et al. 2004, Morozova et al. 2006, Pignataro et al. 2007). Through these studies a large number of alcohol-responsive genes with biological relevance to alcohol-induced disorders have been reported (Table 1.1). Notwithstanding these giant strides achieved from microarray analysis, it suffers from several limitations: a number of other well characterised alcohol-responsive genes such as those encoding neurotransmitter systems are frequently not detected from microarray analysis. An explanation for this is that transcripts encoding neurotransmitter systems tend to have low expression levels compared with other gene categories and a decreased sensitivity to the detection of genes with low abundance is one of the major limitations to microarrays. In addition, recent works have shown that ethanol can induce epigenetic alterations such as acetylation and methylation of histones and other post translational modifications, this is not only opening up a new area of interest in alcohol research but also providing novel insights into actions of ethanol at nucleosomal levels (Spanagel, 2009), however, the disadvantage is that microarrays do not (and cannot be used to) measure these post translational modifications (Luo and Geschwind, 2001).

Table 1.1: Gene expression profiling on human and animal models used in alcohol studies

Study	Subject	Experimental strategy	Statistical criteria for gene selection	Method of validation	Summary of results
Lewohl et al. (2000)	Human frontal cortex	Two case groups each contained an mRNA pool of five alcoholics and an mRNA pool of five controls. Hybridisation to two array types.	Fold change cut of 1.4 used in both the cDNA and oligonucleotide arrays	Cross validation of genes present on the two array types	Coordinated regulation of multiple myelin associated genes most probably responsible for the loss of white matter and demyelinating diseases in alcoholics
Thibault et al. (2000)	Human neural cells (SH-SY5Y)	Two case groups each contained mRNA sample hybridized once for ethanol treated and twice for the control. Hybridisation to two array types.	Fold change arbitrary cut-off of 1.5 used in selecting genes represented by at least ten probe pairs on each of the arrays	Northern blot and reverse transcription-polymerase chain reaction (RT-PCR)	Regulation of genes encoding noradrenaline synthesis and glutathione metabolism and those protecting against apoptosis
Xu et al. (2001)	Mice brain	mRNA pool of four inbred long-sleep and an mRNA pool of four short-sleep mice. Hybridisation to two array types.	Class 1: cut-off of at least 2.0-fold. Class 2: fold of 1.5 or higher and met other conditions including signal to background ratio greater than 2.5, signal intensity greater than 100, and signal size greater than 30% of the spotting area.	Relative quantitative reverse transcription-polymerase chain reaction (RT-PCR)	Detection of 41 differentially expressed genes some of which appears to have biological relevance in mediating differences in ethanol sensitivity

Mayfield et al. (2002)	Human frontal and motor cortex	Three case groups for each brain region; each contained an mRNA pool of five or six alcoholics and an mRNA pool of five or six matching controls. Hybridisation with fluorescent label inversion resulted in 11 successful experiments	Fold change arbitrary cut-off of 1.4 used in at least six of 11 hybridisation experiments	Repeat of hybridisation	Regulation of multiple myelin associated genes most probably responsible to the loss of white matter and demyelinating diseases in alcoholics. Also altered are genes involved in protein trafficking, Ca and cAMP signaling
Daniels & Buck (2002)	Mice hippocampi	Three case groups each contained an mRNA pool of three hippocampi mice for six treatment groups matched for strain, cohort and treatment group (2 chronic EtOH treated with 2 control and 1 acute EtOH treated with 1 control)	Average expression fold change cut-off of at least 1.4 used with standard error of no more than 0.1	Not specified	Altered expression in Janus kinase/signal transduction pathway and the mitogen activated protein kinase pathways
Rimondini et al. (2002)	Rats singulate cortex and amygdale	Two case groups each contained an mRNA pool of three rats for two ethanol-treated brain groups and an mRNA pool of three rats for two control brain groups.	Detected in at least three of the four comparisons with an average expression fold change cut-off of at least 2.0	Not specified	Results show changes in neurotransmission, synaptic plasticity and signal transduction pathways. Chronic exposures and withdrawal lead to marked increase in ethanol voluntary intake

Saito et al. (2002)	Rats hippocampus	mRNA of 3 ethanol-treated animals and 3 matching controls.	t-test, arbitrary cut off of at least 1.5-fold change and the mean expression level of at least 0.37 in either the control or treated group	Not specified	Genes involved in oxidative stress and membrane trafficking were regulated
Sokolov et al. (2003)	Human temporal cortex	mRNA of 11 individual alcoholics and 11 matching individual controls some of which were repeated two to three times in addition	Expression ratio of individual alcoholic and matched control sample and sample direction of change in 10 of 11 alcoholics; one sample t-test	Repeat of hybridisation, real-time PCR	Results demonstrate the implications of ionic homeostasis (Ca regulation) and energy metabolism in ethanol adaptation mechanisms
Kwon et al. (2004)	*C. elegans*	mRNAs obtained from each sample of 7-independent experiments: four sets of 6-h ethanol-treated, two sets of 15 min ethanol-treated, and one set of 30 min ethanol-treated	Modified t-test, arbitrary cut off of at least twofold change and *B* value of at least 0	Northern analysis	Major gene expression involved heat shock protein genes. One gene encoding glutamate receptor was also induced
Iwamoto et al. (2004)	Human PFC	Case group1: mRNA of three individual alcoholics and two controls. Case group 2: mRNA of five individual alcoholics and 3 controls. Control group 3: mRNA of 15 controls	Mann-Whitney *U*-test, arbitrary cut off of at least twofold change	Cross validation of genes, real-time PCR	Remarkable induction of myelin-related genes and molecular chaperones

Treadwell et al. (2004)	Mice whole brain	Two case groups each contained an mRNA pool of five ethanol-treated and an mRNA pool of five controls. Hybridisation to two array types	MAS 5.0 default settings: detected that met statistical criteria in four separate comparisons	reverse transcriptase-polymerase chain reaction (RT-PCR)	Genes involved in stress response, cell signaling gene regulation and homeostasis were identified
Liu et al. (2004)	Human frontal and motor cortex	aRNA of seven alcoholics and seven matching controls, hybridised individually against aRNA of universal reference	Class 1: Detected in at least four more samples of one group compared with the other. Class II: Bayesian posterior probability, detected in at least four samples	Not specified	Results demonstrate the implications of genes encoding metabolism, immune response, cell survival energy production and signal transduction in chronic ethanol response
Morozova et al. (2006)*	*Drosophila*	mRNAs obtained from each sample of 3-independent experiments each contained five replicates of pool of 15 mRNA treated with 0, 1 and 2 ethanol exposure(s)	Probes with absent call removed; significant effect of treatment on gene expression determined using one-way fixed effect ANOVAs of the *Signal* metric and a *post hoc* Tukey tests	Mutant analysis	Several genes involved in stress, olfactory response, metabolic, and transcriptional regulators were identified
Pignataro et al. (2007)*	Mice cultured cortical neurons	mRNA obtained from cultured cortical neurons treated with ethanol or heat	Significant effect of treatment on gene expression determined for any group using one-way ANOVA	real-time PCR, immunoblot analysis	Several genes whose products are involved in synaptic transmission, synapse formation and plasticity or in protein trafficking were revealed

Morozova et al. (2007)*	*Drosophila*	Independently artificially selected alcohol resistant, alcohol sensitive and control (each with replicates) lines generated from 60 isofemale fly lines after 25 generation. mRNAs obtained from each lines contained two replicates of 15 three-to-five day-old virgins males and females totalling 24 samples (six lines × two sexes × two replicates).	Probes with absent call in more half of samples removed; significant effect of treatment on gene expression determined using two-way fixed effect ANOVAs of the *Signal* metric test.	Mutant analysis, cross validation of genes.	Behavioural analysis of 37 mutants corresponding to 35 candidate genes, 32 of which were implicated in ethanol sensitivity.
Urizar et al. (2007)*	*Drosophila* head	Three case groups each containing total mRNA from 60 heads of flies. Control group: mRNA pool of five set of humidified air-treated flies. Rapid tolerance group: mRNA pool of six set of acute EtOH-treated flies. Chronic tolerance group 3: mRNA pool of six set of chronic EtOH-treated flies.	Significant effect of treatment on gene expression determined for any group using one-way ANOVA.	Mutant analysis of one gene named, *homer.*	Results implicate genes encoding transcription factors, signaling proteins, RNA-binding and metabolic enzymes.

Shows a modified summary of the review by Worst and Vrana 2005 and Flatcher-Blader et al. 2006. Papers marked with asterisk were separately included

1.7 Ethanol metabolism

Metabolism is the set of chemical reactions that occurs in living organisms for the maintenance of life. This series of metabolic reactions takes place in an organism upon consumption of substances and as such determines which of these substances it will find useful and which it will find poisonous. In this way, it results in some substances becoming more, and some less, toxic than those originally consumed or ingested. Metabolic reactions are therefore organised into pathways, in which one chemical is transformed into another by a series of enzymes.

Metabolism involves a number of processes, one of which is known as oxidation. Through oxidation ethanol is broken down into harmless substances through a process known as detoxification and subsequently removed from the liver. In this way, alcohol is prevented from accumulating and destroying cells and organs in the body. Over 90% of the absorbed alcohol is metabolised in the body, yielding some 7kcal/g on complete oxidation to CO_2 and H_20. This result in a resultant fall in the body's respiratory quotient (Pawan, 1972). The remaining alcohol in the system is got rid of with another 1% excreted unchanged in the urine, expired air and sweat while the rest is metabolised via other pathways. Until all the alcohol consumed has been metabolised, it is distributed throughout the body (being soluble in both water and lipids), affecting the brain and other tissues.

The main site of alcohol metabolism is the liver; although some other tissues such as kidney, muscle, lung, intestine and brain have been implicated to metabolise smaller quantities (Pawan, 1972). For example, evidence of brain ethanol oxidising properties emerged from rat with the observation that ethanol interacts with brain catalase in vivo (Cohen et al., 1980). Another experiment has documented the presence of ethanol-metabolizing enzymes in human brain with the observation of the metabolism of ethanol to fatty acid ethyl esters. The activities of these enzymes were said to reside in both the gray and white matter of the human brain (Laposata et al., 1987). Other studies have reported the presence of acetaldehyde production within the brain (reviewed in Quertemont et al., 2005). Nevertheless, ethanol metabolism is carried out by three major enzymatic pathways (see Figure 1.3).

The first enzyme system is a rate limiting-step and involves the oxidation of ethanol in the liver to produce acetaldehyde. This reaction is catalysed by the enzyme, alcohol dehydrogenase (ADH) in both man and *Drosophila*. However, unlike the mammalian ADH which is a zinc-containing enzyme, the ADH of *Drosophila* is not a metalloenzyme, and it differs significantly in size and amino acid sequence (Jö rnvall et al., 1984). The breaking down of alcohol into acetaldehyde is regulated by NAD^+ which act as a cofactor and the process occurs chiefly in the soluble cytoplasm of the liver cells. ADH is an enzyme having many different variants (i.e. isoenzymes) and in humans, five different classes with different subunits consisting of 374 amino acids residues and about 10% total amino acid exchanges have been categorised based on their kinetic and structural properties (Agarwal, 2001). These allelic variations in human ADH (and aldehyde dehydrogenase, ALDH) genes appear to contribute not only to differential rates of ethanol clearance in human populations but also to variations in the susceptibility to alcohol dependence and/ or organ damage in response to chronic alcohol consumption (Nagy, 2004).

Another pathway of ethanol metabolism involves the microsomal ethanol-oxidizing system, MEOS, located in the smooth endosplasmic recticulum and involves a cytochrome P450 enzyme (i.e. CYP2E1). This system accounts for the major non-ADH ethanol metabolic pathway in the liver (Lieber, 2000). In man, several polymorphisms of the enzyme cytochrome P450IIE1 (CYP2E1) has been reported (Agarwal, 2001) but with no reported association with susceptibility to alcoholism or alcohol-induced organ damage (Nagy, 2004). CYP2E1 has a low ethanol catalytic efficiency relative to ADH and therefore is responsible for only a small part of total ethanol metabolism.

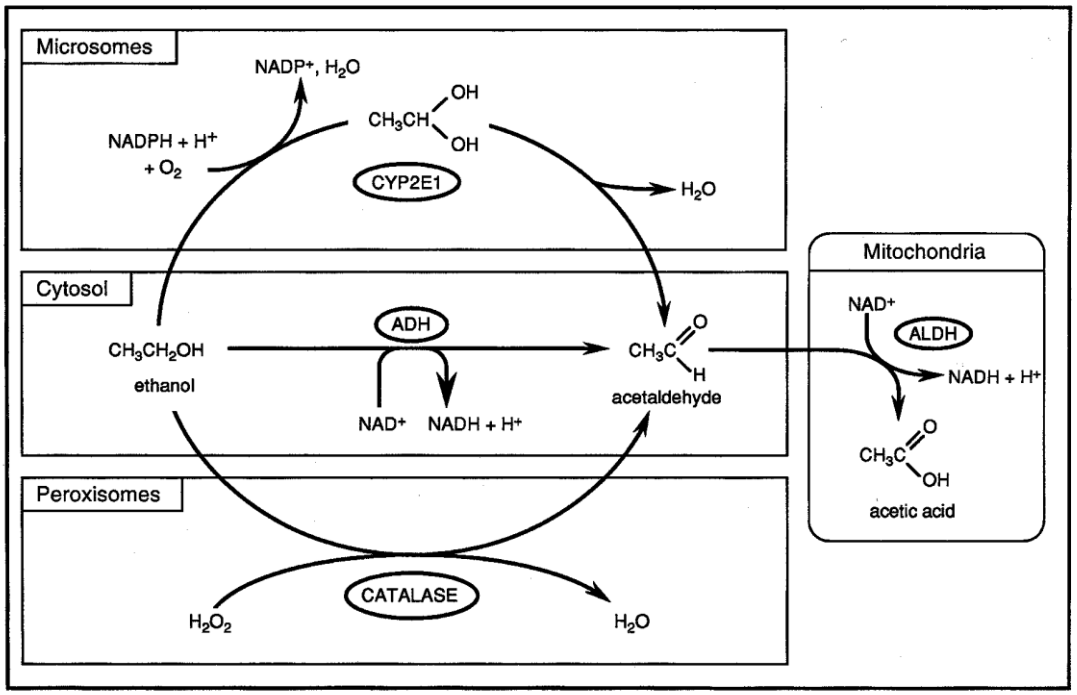


Figure 1.3: Ethanol metabolism. It shows the three pathways involved in the metabolic process. Adapted from: www.benbest.com/health/alcohol.html.

CYP2E1 has been shown to be inducible by ethanol consumption (Montoliu et al., 1994; Lieber, 2004) and such induction appears to contribute to metabolic tolerance to ethanol in human alcoholics (Nagy, 2004). This enzymatic pathway thus assumes an important role in the breaking down of ethanol to acetaldehyde at elevated ethanol concentrations. It also produces ROS such as hydroxyl ethyl, superoxide anion, and hydroxyl radicals, which increase the risk of tissue damage (Wu and Cederbaum, 2005).

The third pathway is the peroxisomal oxidation of ethanol and involves the use of the enzyme catalase. Catalase is capable of oxidizing ethanol in vitro in the presence of a H_2O_2 generating system, such as the enzyme complex NADPH oxidase or the enzyme xanthine oxidase. This however, is quantitatively considered a minor pathway of alcohol oxidation (even less active than MEOS), except in the fasted state (Handler and Thurman 1990).

Unlike in man, ADH metabolises over 90% of ethanol in *Drosophila* to acetaldehyde (Guarnieri and Heberlein, 2003). This ADH is found in the fat bodies and gut in

larvae and fatty acid and gut in adults fruitfully (Geer et al., 1988). Flies have a single gene encoding ADH with its locus under the control of two tandem promoter regions, namely: Proximal (PP) and Distal (DP) promoters which are active at different periods of development (Kapoun et al., 1990) with the proximal promoter being transcribed at a very high level from mid-embryogenesis to the mid-larval third instar and latter diminished and remain low throughout adulthood while the distal promoter is transcribed at a low rate throughout development and at a high rate during adulthood (Savakis et al., 1986). Transcription from the proximal promoter but not distal promoter is upregulated up to five fold in response to a diet that contains 2.5% ethanol (Kapoun et al., 1990). *Drosophila* mutants without ADH activity are fully viable and fertile but are sensitive to exposure to high concentrations of ethanol (> 6%) (Sofer and Martin, 1987). Therefore, it is very unlikely that low ethanol concentration could trigger differential regulation of *Drosophila Adh* gene.

The acetaldehyde formed by any of the three pathways mentioned above, is rapidly metabolised to acetate by aldehyde dehydrogenase (ALDH) in man (but primarily by ADH in *Drosophila*, and only partially by ALDH (Heinstra, et al., 1989)) utilizing NAD as cofactor, and eventually to acetyl-CoA, the Krebs cycle, and other reactions (Figure 1.3).

1.8 Animal models in alcohol research

Animal models involving vertebrates and invertebrates have been used by researchers to study the genetics of alcohol dependence. This is because there is are very high degree of similarity between the DNA sequences of human and other organisms used as models such as primates, rodents, fruit flies or yeast (Philips, 2002). Therefore, any findings linking specific genes or a set of genes with specific alcohol-related physiological changes and behaviours in these organisms can at least to some degree be extended to human alcoholics and vice versa. One example, illustrating this position involved genes encoding the cAMP pathway signaling system which is important for regulating many important processes in the cell. Genetic manipulation of this pathway has been shown to alter ethanol sensitivities in both flies and mice as discussed below (Moore et al., 1998, Wand et al., 2001). In

addition, evidence for a specific role of this pathway in the human addictive brain has been reported (Yamamoto, et al., 2001).

The use of animal models to study genes implicated in ethanol-related physiological changes, thus has many benefits including:

- Fewer ethical boundaries than in human studies
- Higher efficiency for identifying genes underlying human alcohol-seeking behaviour
- The ease with which genetic make-up can be determined and/manipulated (e.g. making homogenous) by the experimenter
- Possible breeding strategies that cannot be performed in humans
- The relative ease with which variables such as environment, drug-intake, and conditional motivators can be controlled.

However, genetic animal models suffered from one principal limitation (among others) that restricts their use to certain, suitable investigations: '*they are simulacra of their more complex human conditions'* (Crabbe et al., 1994), meaning they can only be used to model certain features (out of the whole features) of a diseased condition or behaviour found in humans. Notwithstanding this limitation, individual features of the complex drug-related behaviours and neurobiological responses can be modeled and studied successfully (Crabbe et al., 1994).

1.9 *Drosophila* model in alcohol research

The fruit fly, *Drosophila melanogaster*, is a laboratory tool with an amenable variety of genetic and molecular manipulations that make it very popular organism for study. Flies are easy to raise and maintain, and have short generation time, thereby allowing many experiments to be completed in small time-scales. The *Drosophila* genome has been sequenced (Adams et al., 2000) and well annotated (Drysdale et al., 2005) providing more room for investigation and easy access to information. Flies have well understood genetics with publicly available collections of mutations at single loci (Bellen et al., 2004) and deficiencies covering about 80% of the genome and useful for high resolution mapping, many of which have molecular

defined break points in an isogenic background (Parks et al., 2004). Creating transgenic flies or targeted gene disruption is also relatively simple.

Drosophila models of human diseases are potentially powerful systems for identifying genetic modifiers, therapeutic targets, and drug testing (Mackay and Anholt, 2006). This is because more than 60% of all the genes known to affect human disease have *Drosophila* orthologs, and more than half of all *Drosophila* protein sequences are similar to those of mammals (Rubin et al., 2000). Flies are therefore, good experimental animal for genetics or molecular study of complex traits disorder such as alcoholism and have been successfully used in the past to study the effects of ethanol. They had been used to study susceptibility to ethanol effects by measuring the degree of sensitivity and/ tolerance to the sedative or motor-impairing effects of ethanol (Moore et al., 1998; Scholz et al., 2000).

Flies normally encounter ethanol in their environment as they feed on fermented plant material. As in vertebrates flies have similar enzymes with which to metabolise this ethanol. The role of these enzymes as a critical factor in ethanol metabolism in both flies and humans has been previously discussed (section 1.7). Exposing flies to low concentrations of ethanol triggers locomotor activity, whereas high concentrations induce an intoxicated phenotype similar to human intoxication, characterised by impaired locomotor ability, loss of postural control, sedation, and immobility (Singh and Heberlein, 2000; Wolf et al., 2002).

Ethanol sensitivity and tolerance in *Drosophila* has been measured using a variety of behavioural paradigms. One of these paradigms known as the inebriometer has been used by the Heberlein lab to study aspects of ethanol-induced changes in the nervous system. The inebriometer is a 4-ft long vertical glass column, which contains a series of oblique mesh baffles on which flies can stand. Flies are introduced into the top of the column pre-circulated with standard ethanol vapour. As they lose postural control, they fall through the column. The mean elution time from the column is used as a measure of sensitivity to ethanol intoxication. For measuring tolerance to ethanol, flies obtained from the initial ethanol sensitivity were given some sufficient time to recover from and metabolise the first ethanol

exposure and then re-introduced into the inebriometer. The relative difference between the first and second inebriometer exposure is used as a measure of tolerance to ethanol intoxication (Heberlein, 2000; Scholz et al., 2000). Other behavioural assays used to investigate ethanol response in *Drosophila* include but are not limited to: locomotor tracking systems (Scholz et al., 2000), sedation paradigms (Wen et al., 2005), and recovery paradigms (Berger et al., 2004; Wen et al., 2005; Cowmeadow et al., 2005).

Measuring ethanol-induced behaviours in *Drosophila* can be used to clarify many different aspects of the nature of ethanol. Consequently, studies involving mutant screenings have identified important pathways in the genetic and neural networks that mediate ethanol sensitivity and development of ethanol tolerance in *Drosophila*. One such pathway involved in ethanol sensitivity is known as cyclic adenosine 3, 5-monophosphate (cyclic AMP) signaling pathway and is defined by *cheapdate*, an allele of *amnesiac* (Moore et al., 1998), which encodes a neuropeptide that has been implicated in olfactory memory (Quinn et al., 1979). This pathway is thought to be activated by *amnesiac* neuropeptide (Fanny and Quin, 1995). Another gene named *rutabaga* which encodes a calcium/calmodulin-dependent adenylate cyclase (Moore et al., 1998) and the *Fasciclin II*, an axonal migration and cell adhesion receptor (Cheng et al., 2001), both of which are expressed in the mushroom bodies, have also been implicated in ethanol sensitivity. Flies with reduced cyclic AMP-dependent protein kinase activity display reduced sensitivity to ethanol exposure (Park et al., 2000) and targeted expression of an inhibitor of cyclic AMP-dependent protein kinase to specific regions of the brain using the GAL4-UAS binary expression system implicate a small group of cells located outside the mushroom bodies in reduced ethanol sensitivity to ethanol-induced locomotor impairments (Rodan et al., 2002).

On ethanol tolerance, repeated exposure to ethanol in *Drosophila* has been reported to induce ethanol tolerance (Scholz et al., 2000). Indeed, a single exposure to ethanol has the capacity to induce lower sensitivity to a subsequent ethanol exposure (Scholz et al., 2000). Ethanol tolerance has been reported to be mediated at least in part by two pathways in *Drosophila* neural circuits: a stress pathway

defined by the *hangover* (*hang*) gene which encodes a nucleic acid-binding zinc finger protein. The other pathway is defined by *Tbh* gene and involves octopamine, the neurotransmitter homolog of noradrenaline in vertebrates (Monastirioti et al., 1996; Roeder, 1999). Mutant flies in which *hang* or *Tbh* expression is abolished displayed reduced ethanol tolerance following ethanol exposure. However, ethanol tolerance is almost completely abolished in *Tbh hang* mutant double recombinant flies (Scholz et al., 2005).

Finally, *Drosophila* through the discovery of LUSH protein (an odorant binding protein) has made it possible to model how transmembrane residues can form specific protein-binding site for ethanol (Spanagel, 2009). LUSH's structure reveals a specific ethanol-binding site and LUSH's mutant flies display abnormal attraction towards high ethanol concentration (Spanagel, 2009). This study therefore combines a whole genome approach using microarray and mutant analysis, and a behavioural genetics approach to identify *Drosophila* genes with altered ethanol sensitivity and or tolerance. This is aimed at identifying an interactive network of genes that may be fundamental for the understanding of the mechanisms governing alcohol dependence in man. Brief descriptions of the list of genes derived from microarray data whose mutant genotypes were assayed for ethanol sensitivity and tolerance using behavioural methodologies are given in the next section.

1.10 Selected Genes of interests

The 7 selected genes with altered expression from the microarray data fall into 3 of the pathways earlier discussed in section 1.5. These are signal transduction (*Axn*, *hop, ana*), stress (*hiw*, *hsp26*, *hsp83*, *mbf1*) and ubiquitination (*hiw*, *hsp26*) pathways. It should be noted that the selection of these genes were based on a number of conditions described in section 3.3.3 (a) of Chapter 3.

Anachronism: One of the genes downregulated in the list of regulated genes identified from the microarray data analysis of acute ethanol exposure in *Drosophila* is *anachronism* (*ana). ana* is a glycoprotein secreted by glial cells and is involved in neurogenesis. The *ana* mutation was identified in a *P* element mutagenesis via a histological screening for defects in the organisation of the adult *Drosophila* optic

lobes (Ebens et al. 1993). Evidence from molecular and biochemical analyses revealed that *ana* gene encoding a novel glycoprotein is secreted by glial cells which neighbour neuroblasts that begin divisions prematurely in an *ana* mutant (Park et al., 1997). The *ana* glycoprotein contains a single long open reading frame encoding a 474 amino acid polypeptide with C terminal domain containing an unusually high concentration of histidine residues and six potential N-linked glycosylation sites (Ebens et al., 1993). *ana* is expressed in the glia and a mutation affecting *ana* transcriptional unit results in precocious optic lobe development and in *ana* mutants quiescent postembryonic central brain and optic lobe cells precociously enter S phase (DNA synthesis) and persist into adulthood (Ebens et al., 1993).

ana null mutants (ana^1 and ana^9) third instar larvae have been shown to display reduced olfactory sensitivity to several odorants including ethyl acetate compared to controls while no significant olfactory phenotype was seen in the trials of ana^9 and Oregon R adults with ethyl acetate at several concentrations in a T-maze behavioural paradigm (Park et al. 1995). Thus, this role of *ana* in a behavioural response to ethyl acetate suggested a possible role in other behavioural phenotypes. In addition, a role for glia in the responses to drugs of abuse including alcohol in both *Drosophila* and mammals has been extensively reviewed (Bainton et al., 2005, Haydon et al., 2009). Importantly, *ana* may also play a role in circadian rhythms (Claridge-Chang et al. 2001).

Homozygous *ana* null mutant flies do not live long after eclosion under crowded conditions in competition with wild-type siblings and they display a variable phenotype, ranging from misrouting of fibre tracts to massive disorganisation of the adult optic lobes (Ebens et al. 1993). In this report, ana^1 loss-of-function allele containing a single P element insertion in the 4^{th} intron of the *ana* gene (Figure 1.4) was used.

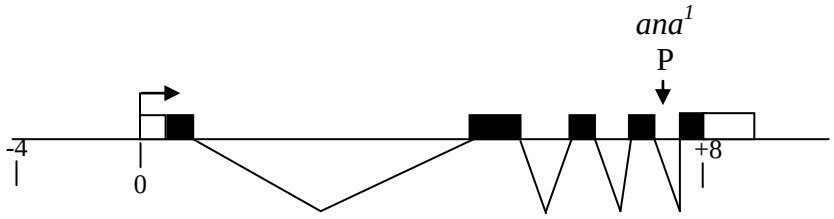


Figure 1.4: Genomic structure of *ana*[1] allele. The intron-exon structure is shown with the P element in *ana*[1] mapping to the last intron. Coordinates are in kilobases, exons are designated in rectangles, with translated regions in closed boxes (shaded black) and un-translated regions in open box (white box). The arrow indicates the transcription initiation site. Diagram re-drawn from Ebens et al., 1993.

Axin: *Axin* is upregulated from the microarray list of differentially expressed genes. It is a negative regulator of wingless signaling pathway known to be involved in cell-cell signaling in many developmental processes (Logan and Nusse, 2004). No drug related behavioural phenotype was reported for this gene. However, the gene encodes Wnt signaling molecules known not only to be required for axon guidance but are also involved in neuronal migration, synaptic differentiation and dendritic morphogenesis (Fradkin et al., 2005). Interestingly, the role of axon guidance in alcohol response has been recently documented (Lindsley et al., 2006). Importantly, the role of axon guidance genes in shaping an individual's behavioural response to suicidality has also been reported (Sokolowiski et al., 2009).

Drosophila Axin (*D-Axin*) was identified using two different approaches: Hamada et al., (1999) performed a yeast two-hybrid screen of a *Drosophila* embryonic cDNA library using the Armdillo repeat domain of Arm as target and identified *D-Axn* as an Arm-interacting protein. Willert et al., (1999) by searching the EST database with the protein sequence of mouse Axin, identified an EST with significant homology to the DIX (a domain similar between Axin and Dishevelled) domain of Axin and used it to isolate the full-length clone from an embryonic *Drosophila* cDNA library. Two D-Axin forms have therefore been identified (Hamada et al., 1999; Willert et al., 1999) with their sequence analysis showing that they are identical but differ only in the serine residues at amino acid position 644 and 645 which was thought to reflect naturally occurring polymorphisms in the Axin gene (Willert et al., 1999). *D-Axin* is expressed ubiquitously throughout development

(Hamada et al., 1999) and a P element insertion near the beginning of the *D-Axin* gene has been shown to disrupt expression of the gene to produce a loss-of-function *D-Axin* allele, an observation also seen with RNAi experiments (Willert et al., 1999).

Axn knockout produced phenotypes that are similar to the overexpression of the *Drosophila* Wnt gene wg while overexpression of *Axin* produces phenotype similar to loss of wg (Willert et al., 1999). Like the vertebrate Axn, *D-Axin* has also been shown to interact with Armadillo and Zeste-white 3 (Willert et al., 1999) and also contain an RGS domain found in a family of proteins that regulate G-proteins, near the amino terminus (amino acids 51 to 171) (Hamada et al., 1999). In this report, the $Axn^{EY10228}$ lethal insertion allele containing a single P element insertion near the beginning of the untranslated 1st exon of the *Axin* gene (Figure 1.5) was used.

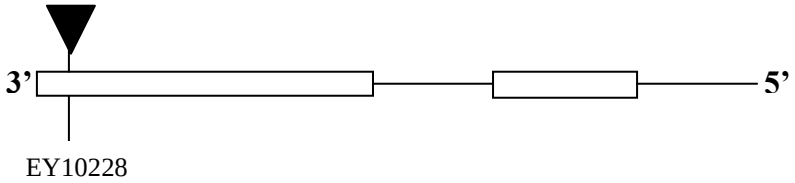


Figure 1.5: Genomic structure of $Axn^{EY10228}$ allele. The exon structure is shown with the P element in $Axn^{EY10228}$ mapping to the first un-translated region.

Highwire: *Highwire* (*hiw*) is upregulated from the microarray list of differentially expressed genes. *Hiw* is a protein binding gene involved in the negative regulation of synaptic growth at the *Drosophila* neuromuscular junction (NMJ). *Drosophila hiw* was identified in behavioural screen of the X-chromosome conducted to detect walking-defective mutants (Wan et al., 2000). *Hiw* encodes a large neuronal protein of 5233 amino acids with evolutionary conserved structure and function (Wu et al., 2005) consisting of an N-terminal guanine-nucleotide exchange factor-like domain, two PHR repeats of unknown function, and a C-terminal RING finger domain that is a putative E3 ubiquitin ligase domain. Hiw transcript is abundant in neurons and its proteins appear to localise to the synapses (Wu et al., 2005).

A mutation affecting the *hiw* gene leads to synaptic sprouting and overgrowth and deficits in neurotransmitter release (Wan et al., 2000; Wu et al., 2005). In addition, a *hiw* E3 ubiquitin ligase domain has been shown to be required for *hiw* function

(Wu et al., 2005). *Drosophila hiw* is homologous to a *C* elegans protein encoded by rpm-1 gene, mouse protein called Phr and a human protein called Pam (protein associated with myc) (Wan et al., 2000). No drug related behavioural phenotype has been reported for this gene. However, a role for ubiquitin in ethanol response has recently been reported: two yeast mutant strains- yeast ubiquitin ligase, Rsp5 and ubiquitin-conjugating enzyme, Ubc4 have been implicated in increased ethanol sensitivity (Hirashi et al., 2009). Given that evolutionary conservation of functions usually exist among genes from different organisms, the possibility of similar behavioural response in *Drosophila* gene encoding an ubiquitin ligase further support the choice of *hiw* as an interesting gene for testing.

Drosophila hiw appears to mediate many biological processes including cellular stress responses and neuroprotection as a link between autophagy, a lysosome-dependent degradation mechanism and *hiw*-ubiquitin mediated synaptic growth and plasticity has been suggested (Shen and Gatnezky, 2009). Thus, the involvement of *hiw* in both stress and ubiquitination pathways also make it intriguing to speculate on a role for this gene in alcohol response. In this report, three *hiw* alleles including one null, hiw^{ND8} were used (Figure 1.6).

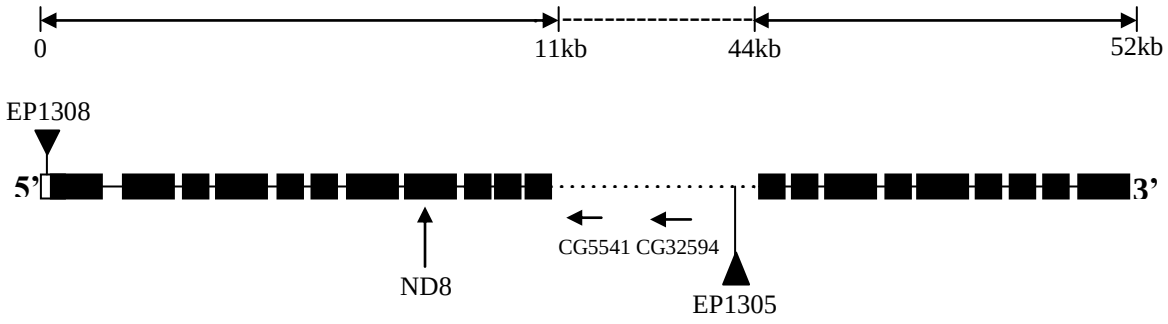


Figure 1.6: Genomic structure of *hiw* alleles. The intron-exon structure is shown with the P element in hiw^{EP1308} mapping to the 5' untranslated region of the first exon while the second P element in hiw^{EP1305} inserts at the 3' end of the large intron. The null hiw^{ND8} allele is a nonsense mutation at amino acid 1930. Coordinates in kilobases are shown above, exons are designated in rectangles, with translated regions in closed boxes (shaded black) and un-translated regions in open box (white box). Two genes, *CG5541* and *G32594*, are located in large intron in dotted line, which spans ~33kb. Diagram re-drawn from Wan et al 2000 and Wu et al., 2005.

Hopscotch: hop is upregulated from the microarray list of differentially expressed genes and it is known to be involved in JAK/STAT signaling pathway. *Drosophila hop* was identified as a mutation with specific maternal effects on embryonic segmentation (Perrimon et al., 1989). It is an X-linked locus that maps to chromosomal bands 10B6-8 and with 27 known loss-of-function mutations (Binari and Perrimon 1994). *hop* is one of more than 50 *Drosophila* oncogenes, that is, genes that cause tumors with a mutation affecting *hop* gene defined by *Tumorous-lethal (Tum-l)* shown to cause formation of melanotic tumors and proliferative defects in larval blood cells (Harrison et al., 1995). Evidence from available databases indicates that *hop* encodes a protein with significant homology at its carboxyl terminus to the catalytic domain of tyrosine kinases (Binari and Perrimon 1994). HOP protein contain a region of internal homology similar to that found in members of Jak family of tyrosine kinases, indicating that *hop* belongs to a member of the Janus family of non-receptor tyrosine kinases (Binari and Perrimon 1994). *hop* is expressed ubiquitously throughout development (Binari and Perrimon 1994) and is required to promote the proliferation and/or survival of the eye imaginal disc cells (Luo et al., 1999).

The *hop* gene has not been previously implicated in ethanol response in *Drosophila*. However, the role of tyrosine kinase in alcohol response has been recently reported in mice: mice deficient for the intracellular protein Fyn tyrosine kinase have been reported to show increased alcohol sensitivity and lack of tolerance to the effects of ethanol (Cowen et al., 2003).

The two *hop* mutants hop^{27} and hop^{25} used in this report are recessive lethal with non-viable males. Molecular characterisation of the *hop* mutations have shown that hop^{27} (also known as hop^{M4}) is located in the kinase domain while hop^{25} (also known as hop^{MSV1}) is located in JH6 domain as determined from the HOP nucleic acid sequence for these two mutations (Luo et al., 1999). The mutations in these two *hop* mutants are chemically induced resulting in point mutations (hop^{25} is induced by EMS while hop^{27} by ENS). The genomic structure of these two *hop* alleles is shown below.

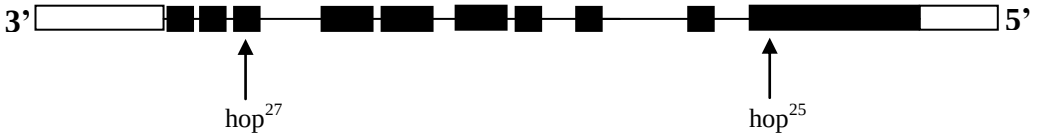


Figure 1.7: Genomic structure of *hop* alleles. The intron-exon structure is shown with the EMS mutation in *hop*^{25} mapping to the 11th exon while the ENS mutation in *hop*^{27} maps to the 4th exon. Exons are designated in rectangles, with translated regions in closed boxes (shaded black) and un-translated regions in open box (white box).

Heat shock protein 26: *hsp26* is the most highly upregulated gene from the microarray data analysis and it is a protein coding gene involved in protein binding. *hsp26* encodes a conserved protein family containing 2 domains, the highly conserved C-terminal region and an N-terminal domain which exhibits sequence divergence ranging from 12 to 40 kDa in different organisms (Haslbeck et al., 1999). Unlike higher Hsps, the binding and release of substrates in *hsp26* did not require either ATP binding or ATP hydrolysis (Jakob et al., 1993). It has been shown to be a temperature regulated chaperone (Haslbeck et al., 1999) and it is involved in aging and oxidative stress (Wang et al. 2004; Liao et al. 2008). *hsp26* has been shown to be expressed in the neurocytes of the brain and the thoracic ganglion (Marin et al., 1993).

Hsp26 is transcribed during certain developmental stages in the absence of heat shock (Sirotkin and Davidson, 1982) and it was shown to be expressed with a robust circadian rhythm in the adult *Drosophila* head, as assessed by microarray analysis using high density oligonucleotide arrays with probe generated during three 12-point time course experiments over the course of 6 days (Claridge-Chang et al., 2001), indicating a role in sleep homeostasis. Though *Drosophila hsp26* gene has not been well characterised and its role in ethanol response has not been reported, the usually high fold change for this gene, its expression in the neurons of the brain and its reported role in oxidative stress suggest that it may be involved in neuronal adaptation to ethanol. In this report, two *hsp26* alleles were used (Figure 1.8).

Figure 1.8: Genomic structure of *hsp26* alleles. The intron-exon structure is shown with the P element in both alleles $hsp26^{EY10556}$ and $hsp26^{KG02786}$ mapping to the same position near the 5' end of the untranslated exon. Exons are designated in rectangles, with translated regions in closed boxes (shaded black) and un-translated regions in open box (while box).

Heat shock protein 83: This gene was selected because of the known role of its mammalian homolog, Hsp90, in ethanol response: an increase in the transcription of Hsp90 in cultured mouse cortical neurons exposed to an acute dose of ethanol has been reported (Pignataro et al., 2007). *hsp83* is upregulated following acute ethanol exposure in the *Drosophila* head. *Drosophila Hsp83* mutations were recovered in screens designed to identify enhancers of *Sevenless* and suppressors of *Raf* and the mutations have been shown to be highly pleiotropic (Yue et al., 1998). The gene encodes the highly conserved Hsp90 protein. Consistent with the highly conserved nature of the Hsp90 protein, it was reported that the human Hsp90 and *Drosophila* Hsp90 proteins rescue Hsp90-deficient *S. cerevisiae* and supports their rapid growth (Yu et al., 1998). Hsp90 protein domain structure contains an N-terminal (ATPase domain) crystal structure corresponding to approximately the first 225 amino acids on the *Drosophila* sequence, a middle domain corresponding approximately to 271-258 amino acids in the *Drosophila* sequence and the C-terminal region containing a dimerisation activity and corresponds to *Drosophila* residues 596-677 (Yu et al., 1998; Song et al., 2007).

Two antimorphic mutations in the *hsp83* gene, encoding a *Drosophila* homologue of Hsp90 protein have been shown to act as dominant enhancers of a hypomorphic raf allele, raf^{HM7} and results in the reduced expression of a wild-type Raf kinase, suggesting that a role for *hsp83* in Raf-mediated signal transduction in *Drosophila* (van der Straten et al., 1997). In *Drosophila*, *hsp83* gene has been shown to impair

germline development (Yue et al., 1998) and play a role in sleep homeostasis (Shaw et al., 2002).

All known *hsp83* mutations are recessive lethal with only *scratch* (*hsp83*^{08445}) that is homozygous viable, female fertile but male sterile (Castrillon et al., 1993). In this report, the *scratch* hypomorphic allele containing P element insertion in the intron located ~60bp from the junction of the 1st exon and the intron and *hsp83*^{e6A}, an EMS mutation in the position of the amino acid exchanges S592F of the C-terminal domain (Figure 1.9), were used.

Figure 1.9: Genomic structure of *hsp83* alleles. The intron-exon structure is shown with the P element in the *scratch* mapping to the first intron while the EMS mutation in *hsp83*^{e6A} maps to the second translated exon. Diagram modified from van der Straten et al., 1997 and Yue et al., 1998.

Multiprotein bridging factor 1: *mbf1* is upregulated following acute ethanol exposure in the *Drosophila* head and it is a stress response protein involved in transcriptional regulation. The gene was first identified from the silkmoth as a co-factor necessary for transcriptional activation in vitro by a nuclear receptor fushi tarazu transcription factor 1 (FTZ-F1) and is shown to connect a regulatory factor and TATA element-binding protein (TBP) (Takemaru et al. 1997). The MBF1 genomic sequence is highly conserved containing 2 functional domains involving a C-terminal half and a N-terminal half and with the *Drosophila mbf1* encoding a predicted cytoplasmic protein of 145 amino acids with 44, 64 and 83% identify to MBF1 from yeast, human and silkmoth, respectively (Liu et al., 2003). *Drosophila mbf1* is expressed at a high level in the central nervous system, imaginal discs and gonads (Jindra et al., 2004). MBF1 appears to be involved in Ca^{2+}-induced gene activation, stress response, homoeostasis and longevity and oxidative stress (Liu et al., 2003; Jindra et al., 2004) thus suggesting its likely role in ethanol response.

In this report, *mbf1*^{2} loss-of-function allele containing a single P element insertion in 21bp upstream of the 1st exon of the *mbf1* gene (Figure 1.10) was used.

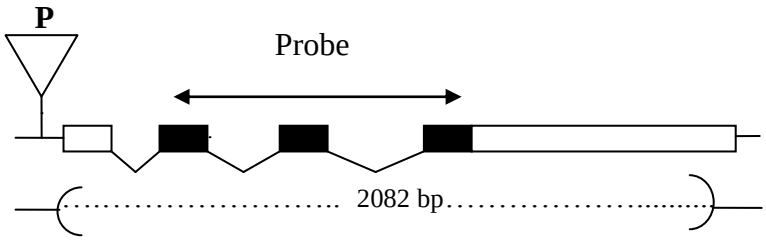


Figure 1.10: Genomic structure of *mbf1*^{2} allele. The intron-exon structure is shown with the P element in *mbf1*^{2} mapping to the first intron. Exons are designated in rectangles, with translated regions in closed boxes (shaded black) and un-translated regions in open box (while box). Diagram re-drawn from Jindra et al., 2004.

1.11 Investigation Summary

This thesis investigated gene regulation in *Drosophila melanogaster* in response to an acute dose of ethanol. The unpublished time course microarray data that were previously generated were used to depict transcriptional changes induced by an acute dose of ethanol at various time points. First, the analysis of the 2 h time point of these microarray data led to the construction of the list of genes whose mutant genotypes were validated using behavioural and genetics methodologies. Further analysis on these data led to the identification of relevant biological pathways and interaction networks of genes in alcohol response. In addition, analysis of the whole time course microarray data revealed the patterns of ethanol-evoked transcriptional regulation within the set of identified genes. In order to accomplish this task, a suitable statistical method was applied to the raw microarray data for differential expression and the list of genes obtained mined with the appropriate bioinformatics tools. Further, suitable behavioural assays for validating some of the candidate genes obtained from the 2 h microarray analysis were adopted, modified and employed to test the behaviour of these genes to ethanol response via mutant analysis. Thus, behavioural paradigms measuring quantifiable variables such as the duration of flies' activity when faced with ethanol stress and the rate of flies' recovery from a sedating dose of ethanol were chosen and used in the mutant analysis.

From the 155 genes identified from the 2 h microarray analysis, 7 genes offering suitable and viable alleles were selected and investigated further. Validation testing with simple behavioural assays revealed the possible roles of these genes in relation to alcohol response. Finally, 2 genes, *heat shock protein 26* and *highwire* were identified as offering possible importance in the pathways governing ethanol tolerance and sensitivity respectively. These two genes were investigated further by individually manipulating them in different regions of the *Drosophila* brain and tested for ethanol sensitivity and/tolerance using sedation and recovery assays; two behavioural instruments for measuring the fly's resistance to and recovery from ethanol sedation respectively. The findings from this investigations form the basis for the conclusions drawn about whether these genes clearly contribute novel insights into brain mechanisms of acute ethanol action.

The goals of this research were to combine bioinformatics, behavioural and genetic approaches to identify genes that may be fundamental to alcohol dependence. Future work will be directed by the findings and recommendations made in this thesis. For instance, the gene list produced from the time course analysis remains to be fully validated while many interesting questions posed in this area are yet to be investigated further. Such could provide useful model for future investigation. Finally, the methodical approach used in the analysis of the Affymetrix microarray data could be used for future work in expression analysis.

Chapter 2.

Experimental Materials and Methods.

2 Experimental Materials and Methods

2.1 General fly handling and husbandry

Flies were grown and maintained at 18°C in vials measuring 9.5cm x 2.5cm diameter or bottles measuring 14cm x 5cm average diameter and stoppered with cotton wool or paper lid respectively and contained an approximate food depth of 3cm in each. The fly food is a standard maize meal food (sprinkled with a small amount of dried bakers yeast obtained from DCL Yeast Ltd, Surrey, UK) in the following proportions:

Ingredients:

Agar	40g
Maize meal	350g
Soya meal	45g
Yeast	82g
Sucrose	185g
Malt extract	370g
Nipagin 10%w/v	82mls
Propionic acid	25mls
Tap water	5725ml

All flies used in experiments were between 2-5 days old to reduce age- related behavioural effects and all assay-specific experiments started at the same time of the day to reduce errors due to variances in results that may be caused by circadian rhythms. The flies used in these experiments were male (this is taking into consideration the need to remove any effect due to possible sexual dimorphism and reduce size discrepancy between individuals) but where it was not possible to generate viable males, female flies were used. Flies were sorted under CO_2 anaesthesia at least 24 hours prior to the start of the experiments to allow recovery. All ethanol used for the inebriometer exposures was 99.86% v/v minimum ('Absolute Alcohol 100'), supplied by Hayman Limited, Essex, UK and in the case of the sedation and recovery assay, HPLC grade absolute ethanol with flash point 8° supplied by Rathburn Chemicals Ltd, Walkerburn, Scotland, UK was used.

2.2 Fly stocks and Genetics

The general fly strains and their genetics annotations are given below. The two control strains of genotypes w^+*; Iso2C; Iso 3I* and w^+*; Iso2A; Iso 3A* were both isogenised on the second and third chromosomes and reported to behave similarly to the commonly used Canton-S stock in a range of behavioural tests (Sharma *et al.*, 2005). These flies were used as the wild-type stocks.

Table 2.1: Fly stocks with their annotations

Stock/Line	Genotypes	Received	Stock Information	FC	Notes
2447	*w^{+}; Iso2C; Iso 3I*	Dec 2007	From Cahir O'Kane	†	Control
2451	*w^{+}; Iso2A; Iso 3A*	Dec 2007	From Cahir O'Kane	†	Control
hop^{27}	*$y^{1}hop^{27}$/Basc*	Jun 2008	Bloomington 8493	2.24	Recessive lethal; non-viable male
hop^{25}	*$y^{1}hop^{25}$/Basc*	Jun 2008	Bloomington 8494		Recessive lethal; non-viable male
$hsp83^{08445}$	*P{PZ}$Hsp83^{08445}$ ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}*	Jun 2008	Bloomington 11797	5.73	Male sterile
$hsp83^{e6A}$	*w^{*};$Hsp83^{e6A}$/TM6B,Tb^{1}*	Jun 2008	Bloomington 5695		Recessive lethal
mbf 1^{2}	*mbf 1^{2}*	Jun 2008	From Hirose	2.07	3rd chromosome
mbf 1^{+}	*yw;6c; mbf 1^{2}*	Jun 2008	From Hirose		Genomic rescue
hiw^{EP1308}	*w^{1118} P{EP}hiw^{EP1308}*	Oct 2008	Bloomington 11421	1.38	X Chromosome
hiw^{EP1305}	*w^{1118} P{EP}hiw^{EP1305}*	Feb 2009	Bloomington 11420		X Chromosome
hiw^{ND8}	*hiw^{ND8}*	Apr 2009	From DiAntonio		X Chromosome
$hsp26^{EY10556}$	*y^{1} w^{67c23}; P{EPgy2}$Hsp26^{EY10556}$*	Oct 2008	Bloomington 20186	26.54	3rd Chromosome
$hsp26^{KG02786}$	*y^{1} w^{67c23}; P{SUPor-P}$Hsp26^{KG02786}$*	Oct 2008	Bloomington 132132		3rd Chromosome
ana^{1}	*w^{*}; P{A92}ana^{1}/CyO*	Oct 2008	Bloomington 8926	2.17	Homozygous viable
ana^{1}	*P{ana^{+m}}1, w^{*}; P{A92}ana^{1}*	Oct 2008	Bloomington 8927		Genomic rescue
$Axn^{EY10228}$	*y^{1} w^{67c23}; P{EPgy2}$Axn^{EY10228}$/TM3, Sb^{1} Ser^{1}*	Oct 2008	Bloomington 17649	1.58	Recessive lethal
Tbh^{nm18}	*Tbh^{nm18}/FM6I*	Jul 2009	From Monastirioti	†	Hemizygous male viable
C819	*C819*	Jul 2009	From Fly-trap.org	†	R2/R4m ring neuron
201Y	*201Y*	Jul 2009	From Fly-trap.org	†	MB Kenyon cells
OK107	*P{GawB}OK107*	Apr 2009	Bloomington 854	†	MB driver
Repo	*w^{1118}; P{GAL4}repo/TM3, Sb^{1}*	Jul 2009	Bloomington 7415	†	Glial cell driver
elav-GAL4	*P{GawB}$elav^{C155}$*	Apr 2009	Bloomington 458	†	Neuronal driver;
elav-GAL4	*P{GAL4-elav.L}2/CyO*	Jul 2009	Bloomington 8765	†	Neuronal driver
elav-GAL4	*w^{*}; P{GAL4-elav.L}3*	Jul 2009	Bloomington 8760	†	Neuronal driver
UAS-Hiw	*UAS-hiw*	Apr 2009	From DiAntonio	†	2nd Chromosome
UAS-GFP-Hiw	*UAS-GFP-hiw*	Apr 2009	From DiAntonio	†	2nd Chromosome
UAS-Hiw-ΔRING	*UAS-Hiw-ΔRING*	Apr 2009	From DiAntonio	†	2nd Chromosome
UAS-hsp26	*UAS-hsp26(II)*	Apr 2009	From Wang	†	2nd Chromosome

Stock/Line	Flybase Genotypes/Lines	Received	Stock Information	FC	Notes
UAS-GFP(II)	*UAS-GFP(II)*	Jul 2009	From Ian	†	2nd Chromosome
UAS-hiw^{RNAi}	*w^{1118}; P{GD14101}v26998*	Jul 2009	VRDC v26998	†	3rd Chromosome
UAS-hiw^{RNAi}	*w^{1118}; P{GD14101}v28163*	Jul 2009	VRDC v28163	†	2nd Chromosome
UAS-hiw^{RNAi}	*w^{1118}; P{GD14104}v36085*	Jul 2009	VRDC v36085	†	2nd Chromosome
UAS-hsp26^{RNAi}	*w^{1118}; P{GD1273}v6983/TM3*	Jul 2009	VRDC v6983	†	Lethal insertion

Table showing information about all the genes used in behavioural analyses. FC is the GC-RMA Fold Change expression for the seven genes validated via mutant analysis.

2.3 Behavioural Assays

2.3.1 Inebriometer Assay for Ethanol Sensitivity

The inebriometer used in the experiment was built by Georgina Hancock, a former DPhil student in this laboratory (see Hancock, 2005). The inebriometer assay was used to measure ethanol sensitivity as previously described (Moore et al., 1998, Sing and Heberlein, 2000, Hancock, 2005). For each assay, 200 male flies were introduced into the top of the inebriometer column, an apparatus that quantifies the effect of ethanol on postural control. The inebriometer is a 4-foot high glass column containing multiple and uniformly distributed oblique mesh baffles through which ethanol vapour of regulated concentration circulates. Ethanol vapour was delivered to the top cap from air pumped through a litre Quick fit flask filled with 800ml EtOH maintained at 21°C (in a water bath) and 300ml water (unheated) both at 22/min each. The column was pre-equilibrated at a temperature of 21°C for 30 minutes with a mixture of ethanol vapour and humidified air at a ratio of 50/50 ethanol/air. Flies become intoxicated (due to the increase in their internal ethanol concentration), lost postural control (i.e. ability to stand on the mesh baffles) and eventually fell down the column. Flies were collected in a funnel with an Eppendorf attached at the bottom of the 4-foot glass tube and counted at 3-minute intervals. Counting started immediately when flies were introduced into the column. All runs were carried out between 8.30 am and 12 pm. The mean elution time (MET) for each sample population of 200 flies was calculated as the sum of the number of flies eluted at every 3 minutes multiplied by the time of elution in minutes and divided by the total number of flies eluted as given by this equation:

$$\text{MET} = \frac{\sum x_t \bullet t}{N}$$

where x_t is the number of flies eluted at a given time t, t is the time of elution and N the total number of flies eluted

2.3.2 Sedation Assay for Ethanol Sensitivity

The sedation assay previously described (Wen et al., 2005) was modified and used to test some of the candidate genes for alcohol sensitivity. For this assay, twenty active and well fed males (or females in the case of stocks that produced non-viable male) were used for each trial. These flies were selected under CO_2 anaesthesia and allowed to recover for 24 hours before use. 1 ml ethanol solution at 50% concentration was added to a piece of folded Kimwipe tissue (11.4 x 21.5 cm) with

edges sealed by using transparent tape and laid at the bottom of a 180 ml plastic fly bottle. Flies were then transferred immediately into the bottle and the bottle sealed with a paper lid and parafilm. The active flies remained on the top inside the bottle and the sedated flies that dropped to the bottom were counted at 6-min intervals. Counting started immediately flies were introduced into the bottles. The percentage active flies for each time interval of 6 minutes in each sedation experimental run of sample population of 20 flies was calculated as the number of flies active at a given time divided by the total number of flies and multiplied by 100 as given by this formula: % Active Flies = $x_{t/N} \bullet 100$ where x_t is the number of active flies at a given sedation time t and N the total number of sample flies.

The Mean Sedation Time (MST) used as a measure fly's resistance to the sedative effects of ethanol was calculated as the sum of the number of flies sedated at every 6 minutes multiplied by the time of sedation in minute and divided by the total number of flies sedated as given by this equation:

$$MST = \frac{\sum x_t \bullet t}{N}$$

where x_t is the number of flies sedated at a given time t, t is the time of sedation and N the total number of flies sedated.

2.3.3 Recovery Assay for Ethanol Sensitivity

The recovery assay has been previously described (Wen et al., 2005) and was also modified and used to test the alcohol sensitivity of the same selected genes. For the recovery assay, twenty active flies (male or female as applicable) were exposed to ethanol vapour for 12 min in a vial closed with a cotton wool plug, to which 1 ml of 100% ethanol was added slowly to allow ethanol to soak into the plug. After this exposure, all flies tested remained motionless at the bottom of the vial. Subsequently, the ethanol-soaked cotton plug was then replaced with a fresh ethanol-free cotton plug. The number of flies recovered from the ethanol sedation as shown by their climbing and flying activities was counted at 3-min intervals. Counting was started immediately after the old cotton plug was replaced with the new one. The percentage of recovered flies at each time interval in both the mutant and non-mutant strains were calculated. The mean recovery time (MRT) for each sample population of 20 flies was calculated (section 2.5.2) and used as a measure

of the fly's ability to recover from the sedative effects of ethanol. The percentage recovered flies for each time interval of 6 minutes in each recovery experimental run of sample population of 20 flies was calculated as the number of flies recovered from the sedative effect of alcohol at a given time divided by the total number of flies and multiplied by 100 as given by this formula:

% Recovered Flies = $x_{t/N} \bullet 100$ where x_t is the number of recovered flies at a given recovery time t and N the total number of flies used.

The Mean Recovery Time (MRT) used as a measure of fly's ability to recover from the sedative effects of ethanol was calculated as the sum of the number of flies recovered at every 3 minutes multiplied by the time of recovery in minute and divided by the total number of flies recovered as given by this equation:

$MRT = \frac{\sum x_t \bullet t}{N}$ where x_t is the number of flies recovered at a given time t, t is the time of recovery and N the total number of flies recovered.

2.3.4 Tolerance Assay

Sedation assays measuring rapid tolerance were performed essentially as in sedation assay for ethanol sensitivity but after initial exposure (MST1), flies were collected in vials and allowed to recover in a humidified room at 18°C on fresh food. They were then expose to ethanol for a second time. The second exposure (MST2) was initiated exactly 4 h after the start of the first exposure. Tolerance development (i.e. percentage increase in tolerance) was calculated relative to the MST of flies following their first and second exposure in the sedation paradigm using the formula: (MST2– MST1/ MST1 X 100) while the percentage change in tolerance standard error, SE (% CHG), is calculated using the formula:

(MST2/ MST1) X $(SE^2_{MST2}/ MST2^2 + SE^2_{MST1}/MST1^2)^{0.5}$ X 100.

2.3.5 Heat-shock– Ethanol Cross-Tolerance Assay

Flies were incubated in a vial (which has already been incubated at 38°C for 18 hrs prior to heat treatment to allow even distribution of heat in the vial) at 38°C for 3 min in a water bath. After a recovery period of 4 h in an 18°C room, the flies were exposed to ethanol in the sedation paradigm (MST^{hs+}). Tolerance was calculated with respect to flies that were not heat-treated (MST^{hs-}), using the formula:
$(MST_{hs+} - MST_{hs-}/MST_{hs-} \text{ X } 100)$ while the percentage change in cross-tolerance standard error, SE (% CHG), is calculated using the formula:
$(MST_{hs+}/ MST_{hs-}) \text{ X } (SE^2_{hs+} / MST_{hs+}^{\ 2} + SE^2_{hs-}/ MST_{hs-}^{\ 2})^{0.5} \text{ X } 100$.

2.3.6 Rescue Experiments

P[UAS-*hsp26*], P[GAL4-elav]/Cyo and P[GAL4-201Y] transgenic lines were crossed into the $hsp26^{EY10556}$ mutant background to generate P[UAS-*hsp26*]/P[UAS-*hsp26*];$hsp26^{EY10556}/hsp26^{EY10556}$, P[GAL4-elav]/Cyo; $hsp26^{EY10556}/hsp26^{EY10556}$ and P[GAL4-201Y]/P[GAL4-201Y];$hsp26^{EY10556}/hsp26^{EY10556}$ flies. For rescue experiments, male flies were F1 progeny of a genetic cross between $hsp26^{EY10556}$ flies carrying a P[GAL4-elav] or a P[GAL4-201Y] driver and UAS-*hsp26*;$hsp26^{EY10556}$ flies.

2.4 Ethanol Absorption Assay

Flies internal ethanol compositions were determined from whole fly homogenates of 2 flies per samples using the Analox AM-1 Alcohol Analyser (Alcometer). The Alcometer carries out rapid, high performance analysis of alcohol concentrations based on the oxygen consumption of the reaction:

$$EtOH + O_2 \xrightarrow{Alcohol\ Oxidase} Acetaldehyde + H_2O_2.$$

The machine allows testing of plasma, serum or precipitated whole blood, and has been shown in our lab to be suitable for testing appropriately prepared *Drosophila* whole body samples (Hancock, 2005). Analysis time per sample was 20 seconds, allowing ultra-rapid analysis of many repeat experiments.

The followings are the protocol for sample preparation for Analox:

1. Flies were exposed to 12 min ethanol vapour in the recovery assay and culled at 0, 1, 2, 3 or 4 h after exposure.
2. Snap-freeze flies immediately in dry-ice at the appropriate time.

3. Put flies into Eppendorf.
4. Add 20µl PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, in 800 ml of distilled water to 1 litre and adjust to pH 7.4 with HCl).
5. Auto-pestle 60 seconds.
6. Centrifuge at 12,000 rpm for 10 minutes.
7. Remove supernatant into new Eppendorf.
8. Repeat steps 6 and 7 twice more each.
9. Snap-freeze final solution for storage.
10. Defrost thoroughly before testing.

Finally, to calculate fly internal ethanol concentration, the volume of 1 fly equals 2 µL (Moore et al, 1998; Berger et al., 2008) was used.

2.5 Confocal Microscopy

Confocal microscopy was performed using a laser scanning confocal system for verifying GFP expression in the assayed *Drosophila* brain regions. 2-5 days old adult *Drosophila* brains were imaged on the confocal microscope and images scanned using excitation (480 nm) and detection (500-550 nm) filters. The gain was chosen as the maximum gain that did not saturate the signal for each sample studied. A complete z-stack was acquired for each brain sample. Tissues were studied at X20. Images are shown in Appendix A.6.

2.6 Statistical Analysis

For the behavioural data all analyses were carried out using '**SPSS15**' and '**Excel**', all licensed to the University. The raw inebriometer data were fitted to Gaussian curves (assuming normal distribution) using a Line chart in Excel. Other behavioural data (i.e. sedation and recovery data) were first converted to percentages before being fitted into their respective curves (profiles) using the same Line chart in Excel. Bar graphs in Excel were also used to represent the MET, MST and MRT for each specific raw assay data with their respective collated Standard Error of the Mean (SEM), allowing the main features of their profiles to be compared. Experimental repeats data (minimum of 5 repeats) for each assay were assessed for any variations using a one-way analysis of the variance (1-way

ANOVA). Finally, the comparison(s) between experimental groups for both sedation and recovery assays and the differences in MST in the case of tolerance data were analysed using either the Students t-test (two-tailed test assuming equal variance of the mean) or 1-way ANOVA followed by a suitable post-hoc test (i.e. Student Newman Keuls). This post-hoc test was used to clarify the direction of a significant result by carrying out a pair-wise comparison between means, selecting those that form homogenous subsets and relate them in order from the highest to the lowest mean.

Chapter 3.

Gene Expression Analysis.

3 Gene Expression Analysis

This chapter presents a study of the effects of acute ethanol administration on gene expression estimates of *Drosophila* head. Section 3.1 provides a detailed background to the study and discusses why microarrays have been used, section 3.2 explains the raw experimental data and the various statistical and computational data mining approaches and section 3.3 presents an analysis of the data. Finally, section 3.4 summarizes the conclusions of the investigation.

3.1 Introduction

3.1.1 Gene expression profiling

Genes contain the instructions for making the messenger RNA (mRNA) in each cell of an organism. However, what makes each cell different is that not all genes are expressed in any one cell at the same time. This principle of gene expression is based on the Central Dogma of molecular biology illustrated in Figure 3.1.

Expression profiling experiments usually involve measuring the relative amount of mRNA expressed in two or more experimental conditions. This is based on the assumptions that most mRNAs get translated into proteins and that most changes at the mRNA levels suggest changes at the level of the protein encoded by the mRNAs. The use of gene expression profiling (such as microarrays) provides a snapshot of all the transcriptional changes in a biological sample. This high throughput method, unlike other techniques such as Southern and Northern blots that focus on a single gene or limited set of genes, facilitates the discovery of totally novel and unexpected functional roles of genes (Slonim and Yanai, 2009). The power of microarrays have been applied to a range of applications including discovering novel disease subtypes, developing new diagnostics tools and identifying mechanisms of disease or drug response (Slonim and Yanai, 2009).

The aim in this thesis is to identify those genes that are differentially expressed between two treatments. The focus is to deduce gene expression profiles in *Drosophila* exposed to an acute dose of ethanol or water over a number of different time points. The motivation is that the differentially expressed genes between these

two treatments are likely to be fundamental to the development of alcohol dependence in man. Microarray analysis of the data should therefore provide a list of candidate genes which can be characterised further using computational, behavioural and genetic methodologies.

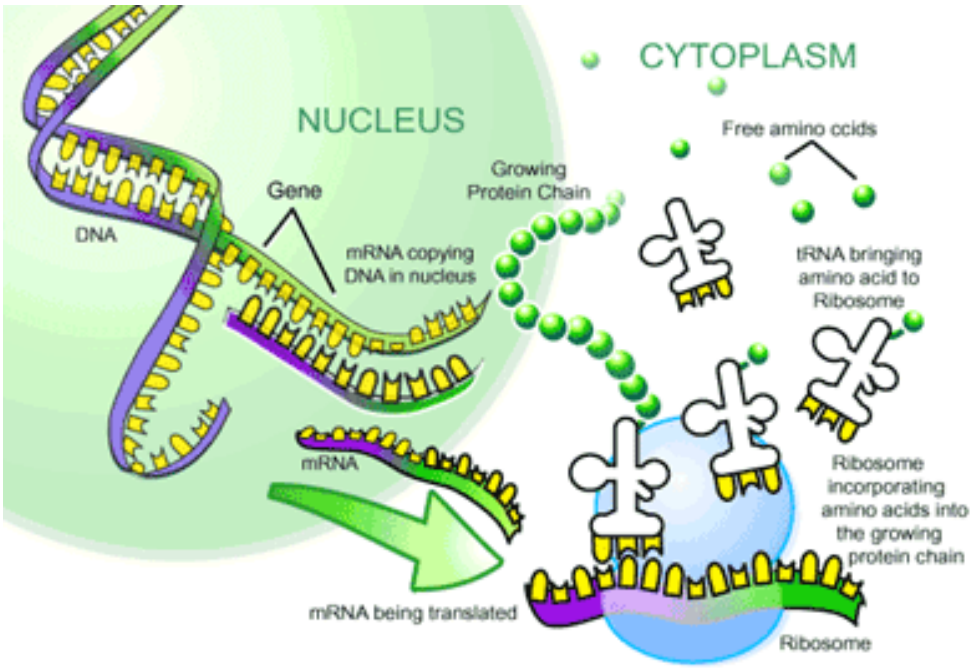


Figure 3.1: The central processes of gene expression: transcription and translation. **Source**: http://www.scq.ubc.ca/wp-content/translation1.gif

3.1.2 Microarray Techniques

A microarray is a microscopic array of large sets of DNA sequences immobilised on solid substrates (Eisen and Brown, 1999). While traditional methods such as western blotting and northern blotting employed for gene expression analysis usually focus on a single gene product or transcript, microarrays constitute an extension of these methods and can be used to monitor thousands of mRNA transcripts in a cell. Microarrays thus give scientists the ability to perform experiments on thousands of genes simultaneously instead of having to gather data on a single gene at a time. Microarrays can be used to monitor the expression levels of practically all genes in an organism simultaneously (Lockhart et al., 1996), and is often referred to as whole-genome expression monitoring. Knowledge of genome-

wide gene expression patterns is required to understand the role of individual genes or gene products in a biological process and has triggered numerous efforts to measure expression levels of thousands of genes from different cellular subsystems across a variety of experimental conditions. These conditions include internal cellular physiology from different cell lines, diverse physiological conditions in an intact organisms, pathological tissue specimens from patients and serial time points following a stimulus to the cell or organism (Raychoudhuri et al., 2001). The technology has thus promised to revolutionise several fields of biological science; from shedding light on the processes of transcriptional regulation to monitoring the effects of clinical drugs in timecourse experiments (Jackson et al., 2005). There are two major types of microarrays, cDNA microarrays and oligo microarrays. This research uses oligo arrays and thus extensive description of these arrays and a brief description of the cDNA arrays, are given in the following paragraphs.

cDNA microarrays: In complementary DNA (cDNA) array experiments, an arrayer device is used to produce the microarrays. This arrayer is a robotic instrument used to spot or print cDNA sequences directly onto a glass or nylon substrate (Stekel, 2003). The cDNA technique uses long probes of hundreds or thousands of base pairs (bps) and is manufactured using deposition technology and can be used to analyse two or more samples simultaneously (Stekel, 2003).

Oligo arrays: Oligonucleotide arrays (oligo arrays) utilise in situ synthetic technologies to produce the microarrays such as the one used by Affymetrix, Inc. to create its commercial microarray product family GeneChip (Stekel, 2003). This technique uses a method similar to that used in the production of solid-state semi-conductors. An array is constructed by building short sequences of RNA (oligonucleotide probes) of about 25 base pairs in length (the length used can vary between 2 to 30 bases) on a solid glass surface. Probes are chemically synthesised from nucleotides at a specific location on the surface of the arrays (Lockhart et al., 1996). Measurement of gene expression involves a light source, synthetic photosensitive protector molecules and lithographic masks allowing the placement of specific nucleotides in preferred locations to form multiple arrays on a single glass surface. Thus, the whole process is called a photolithography and is comprehensively described in (Stekel, 2003). In brief, it involves the use of light to

convert the protective group on the terminal nucleotide into an hydroxyl group to which further bases can be added. The precise location where each probe is synthesised is called a feature. This light is then directed to the appropriate features using masks that allows light to pass to some areas of the array and not to others. Each step of synthesis requires a different mask. One single high density Affymetrix array with typical size 1.28cm x 1.28 cm contains millions of features. At each feature position, the probe is present in millions of copies in order to capture the unknown amount of target molecules with the complementary sequence in the sample. Typically, 11 to 20 probes interrogate a given gene and this collection of probes is known as probeset (Figure 3.2) with about 12000 to 22000 probesets on an array (Affymetrix 2001a, 2004).

On the *Drosophila* Affymetrix 1.0 chips used in this study, each gene is represented by 14 pairs of 25-mer oligonucleotides representing each of the 14,010 transcripts. (Affymetrix 2001a). Each probe pairs consists of a 25-mer oligo known as a perfect match (PM) and the same oligo with a 1bp change in the central position of the oligo known as the mismatch (MM) probe. For example, A is changed to T and C is changed to G and vice versa. The PM reference probe is designed to hybridise only with transcripts (target sequence) from the intended gene (specific hybridisation), i.e. it matches the target sequence exactly. MM probe, on the other hand, is a partner probe that differs from the reference probe at the centre (non-specific hybridisation) (Figure 3.3). The purpose of MM oligos on an Affymetrix chips is to correct for non-specific binding of the mRNA (Affymetrix 2001b). The default adjustment, provided as part of the Affymetrix system, (Zhijin et al., 2004) is based on the difference between perfect match and mismatch (PM-MM) intensities.

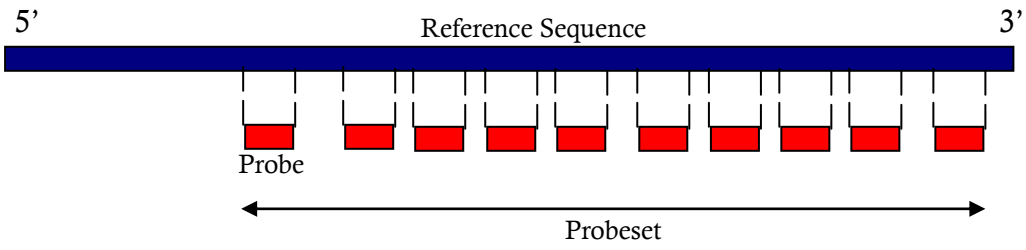


Figure 3.2: Probesets are made up of multiple probes used interrogating the sequence of a particular gene. Redrawn from Bolstad, 2004.

Figure 3.3: Perfect Match and Mismatch Probes

3.1.3 Methods for using Affymetrix microarrays

A typical microarray experiment involves the following steps as illustrated in the Figure 3.4:

1. Isolate RNA from the tissue of interest and prepare fluorescently labelled targets.
2. Hybridise the labelled targets to the microarray
3. Wash, process and scan the microarray
4. Process the resulting image by converting them into numerical values (quantification) - (probe intensities) for statistical analysis.

Figure 3.4 shows the schematic representation of the steps involved in the Affymetrix microarray experiment used in this study. It illustrates a single channel array with the experiment designed to compare the mRNA expression profiles of samples from *Drosophila* head treated with acute ethanol with that of control samples. Each sample is labelled with the same fluorescent dye, but independently hybridized on different arrays (chips).

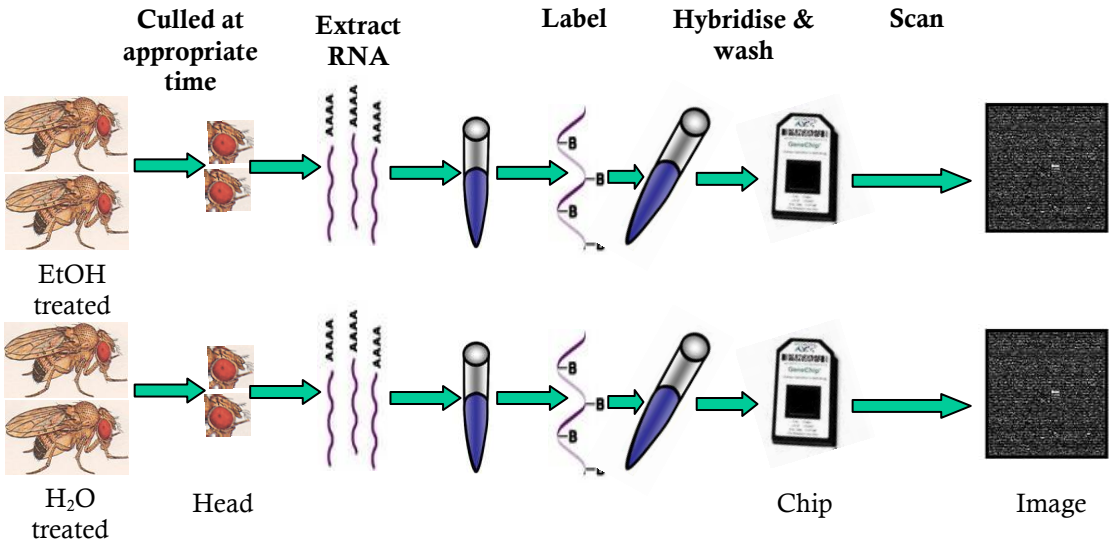


Figure 3.4: The procedure of an Affymetrix microarray experiment on *Drosophila*.

Affymetrix documentation provides the description of how the results from microarray experiments stored in image files are converted into numerical values for statistical analysis (Affymetrix, 2001a, 2004). In brief, an average signal is derived from the signal produced by the scanned labelled and hybridised target for each of the pixels in each probe cell. The pixel information is contained in a data file (*.DAT). The measured intensity values representing the expression level of the related gene and the coordinates on the array for each cell from the data file are stored in a cell intensity file (*.CEL). Each chip thus, corresponds to a CEL file. Affymetrix also provides an array layout definition file (*.CDF), used to store information to a specific type of oligonucleotide array and with all arrays belonging to a given type sharing the same information. The CDF file contains information on the design of a chip indicating which probes belong to which probe-sets. Thus, by looking up the CDF file the intensity (*.CEL) values for each probe-set can be extracted (Affymetrix, 2004).

3.1.4 Overview of the Microarray Data Analysis Process

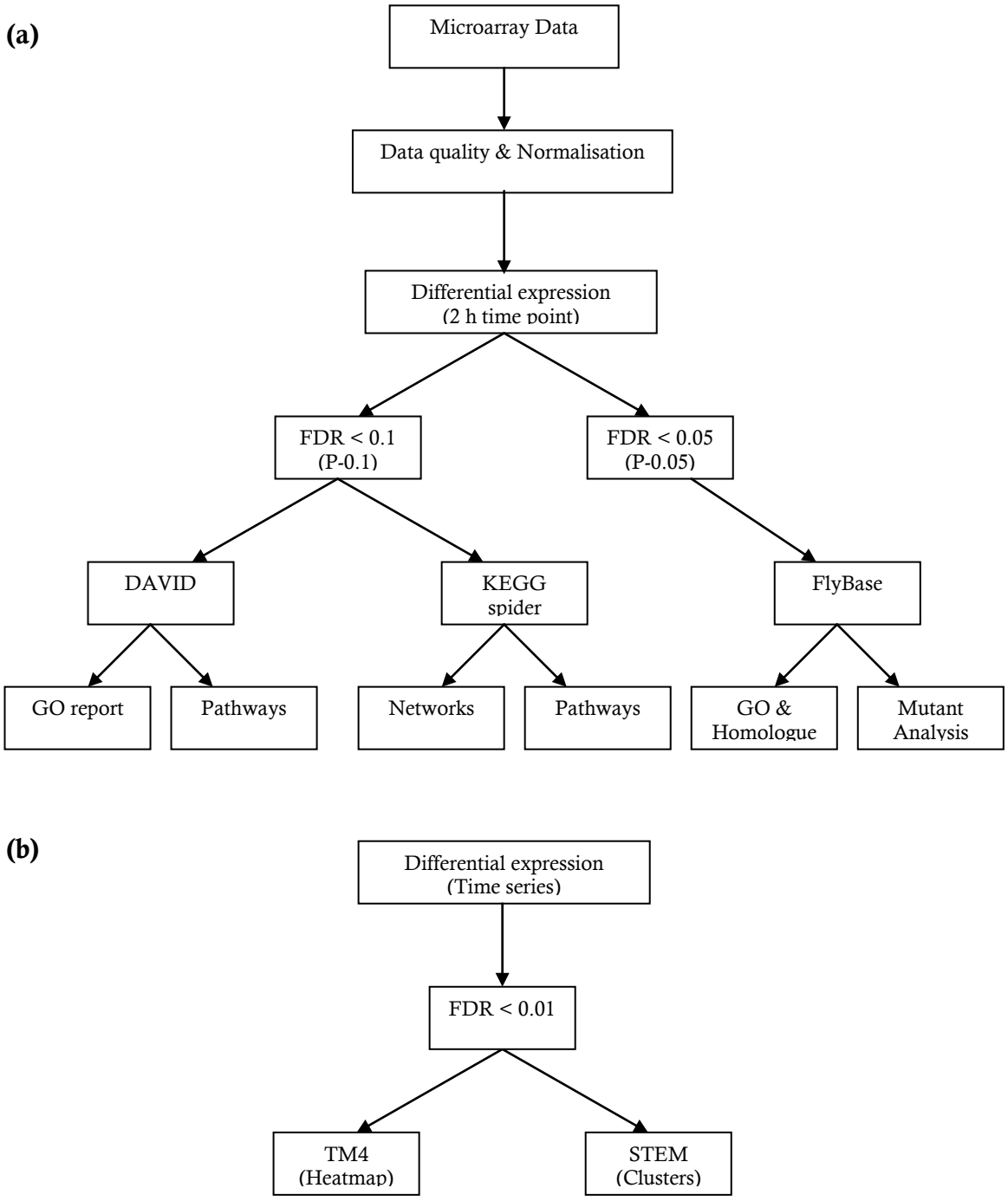


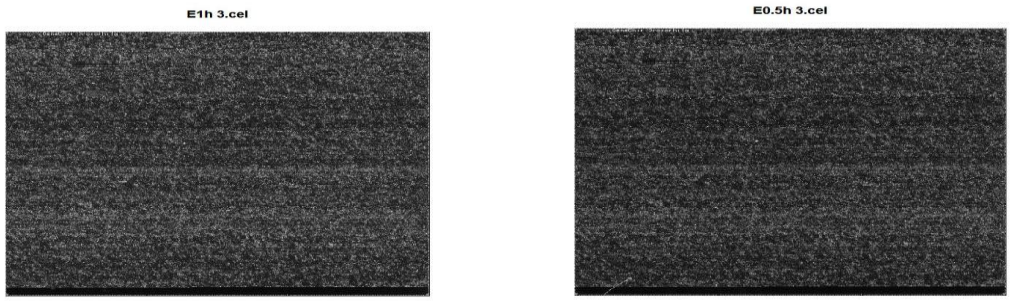
Figure 3.5. The whole microarray data analysis workflow used in this thesis.

3.1.5 Data Quality

Quality assessment of Affymetrix data is required for detecting any chip anomalies and allow for such chips to be removed before statistical tests for differential gene expression are carried out. There are a number of quality assessment steps used in determining the quality of microarray data (Bolstad et al., 2005). The first steps in microarray data pre-processing involve image scanning and spot finding and the selection of good quality spots (Butte, 2002). Improving the reliability of expression measurements starts with proper experimental design such as pooling biological samples before hybridisation to ensure true replicates (Butte, 2002). In addition, several exploratory data quality assessments are available to determine if any anomalies exist in the probe-level data. First, scanned hybridisation images need to be inspected for artefacts such as scratches and bubbles or other non-homogenous patterns in the image plots. Figures 3.6 (a) and (b) show two of the images with no obvious anomalies of the *Drosophila* chips used in this study. Viewing the image plots across all arrays may help to reveal whether one or more arrays might appear abnormal. In this way, a potentially defective array which can be determined based on its colour (appearing lighter or darker than others) or its spatial artifacts (presence of rings, shadows, bubbles not evident in others) may be removed (Bolstad et al., 2005). Next, boxplots and density plots of the probe-level data can be used to determine the existence of potentially defective arrays (Alvord et al., 2007). Thus one look for boxplots that stand out from others, as indicated, for instance by distinctly different ranges or displaced boxes (interquartile ranges, IQR), or density plots that are removed from others, or that display bimodalities, show uniquely different shapes or other abnormalities (Alvord et al., 2007).

Other exploratory plots for quality assessment include the MA plot and RNA degradation plots. The MA plot shows the plot of the difference in log intensities between two microarrays for each probe on each gene (denoted by 'M') against their average (denoted by 'A') (Bolstad et al., 2005). In the case of comparisons involving more than two arrays, a synthetic array is created by taking the probe wise medians across all arrays allowing each microarray to be plotted against the synthetic array (Bolstad et al., 2005). Array quality problems are most apparent in this plot where the loess (locally weighed scatteredplot smoothing) smoother

oscillates wildly or if the variability of the M values appears greater in one or more arrays relative to the others (Bolstad et al., 2005). The RNA degradation plot on the other hand, is used as a measure of assessing RNA integrity and is based on the assumption that when RNA degradation is sufficiently advanced, the PM probe intensities should be systematically elevated at the 3' end of a probe set, when compared to the 5' end (see Alvord et al., 2007).



(a) (b)

Figure 3.6: Pseudo-images of two of the *Drosophila* chips used in this study. In both (a) and (b) an image each for one of the 1hr and 0.5hr time-points replicates respectively is shown.

3.1.6 Normalisation

Microarray data are often coupled with many sources of variations. Irizarry et al. (2003b) explained two sources of variations in high density oligonucleotide arrays- (a) interesting and (b) obscuring variations. Interesting variations are sources of genetic variation between two experimental conditions, for example, high expression of a particular gene or genes may result from a disease process due to variation between diseased and normal tissue. Obscuring sources of variations are variations (in the form of observed expression levels) introduced during sample preparation, manufacture of the arrays or the processing of the arrays (labelling, hybridisation and scanning). Thus, before data from multiple microarray experiment can be pooled into a single analysis the data must first be normalised and corrected for possible sources of obscuring (or technical) variations.

Various methods which include background adjustment, normalisation and summarisation have been proposed for normalising Affymetrix (GeneChip) arrays (Huber et al., 2005). Background adjustment is required to remove the intensity caused by non-specific hybridisation and the noise in the optical detection system. Normalisation is required for removing experimental variation such as different levels of labelling and account for non-specific hybridisation. Essentially, normalisation is used to compare intensity data from multiple arrays. Finally, summarisation is required to calculate expression levels when transcripts are represented by multiple probes (Huber et al., 2005).

Many algorithms are available for normalising GeneChip data and also for calculation of their expression values. The most commonly used are RMA (Irizarry et al., 2003a; Irizarry et al., 2003b), GCRMA (Wu et al., 2004), MAS5.0 or its successor GCOS(Affymetrix, 2001), dChip (Li and Wong, 2001). MAS 5.0 or its successor GCOS is used by Affymetrix systems and makes use of information from only one microarray and also incorporates both the PM and MM probes (Affymetrix, 2001b). In contrast, the model based algorithms involving RMA and dChip incorporate information from multiple microarrays to calculate the expression of a gene by fitting probe response patterns over multiple arrays with a multiplicative model in dChip (Li and Wong, 2001) and an additive model in RMA (Irizarry et al., 2003ab). These fitted models are used to detect abnormal probes, which are subsequently excluded from gene expression calculation (Millenaar, et al., 2006). The GCRMA algorithm makes use of two model types, namely GC and RMA (Wu and Irizarry, 2004) thereby using a method of background adjustment that incorporates the physical model of the GC content of the probe. It should be noted that while MAS5.0 and dChip in the PMMM mode use both PM and MM signals to calculate gene expression, dChip PM mode (Li and Wong, 2001), RMA (Irizarry et al., 2003a; Irizarry et al., 2003b) and GCRMA (Wu and Irizarry, 2004) only use the PM information to calculate gene expression. It should be noted that the MM data in GCRMA is used for modelling the background effect and hence is not entirely discarded (Wu and Irizarry, 2004).

Many studies have been carried out to bench mark the effectiveness of these normalisation algorithms (Bolstad et al., 2003; Verhaak et al., 2006; Qin et al.,

2006; Millenaar, et al., 2006). However, no definite conclusions have been reached on which of the algorithms are most effective for normalisation and calculation of gene expression. This is because these algorithms measure gene expression and normalise the data in different ways, and it appears that the effectiveness of these methods can be influenced by the size and the type of data set. For instance the effect of the four pre-processing strategies involving dChip, RMA, GCRMA and MAS5.0 on expression level measurements, detection of differential expression, cluster analysis, and classification of samples have been assessed (Verhaak, et al., 2006). The sample used involved gene-expression data of 285 patients with Acute Myeloid Leukaemia (AML) and 42 samples of tumour tissue of the embryonic central nervous system (CNS). It was found that in most cases, the choice of these algorithms has a relatively small influence on the final analysis outcome of large microarray data (AML dataset), but has a more profound effect on the outcome of the small microarray data (CNS dataset) (Verhaak, et al., 2006). In addition, another study evaluated the effect of different processing methods on oligonucleotide arrays via quantitative real-time PCR and found no advantage on the choice of one algorithm to the other (Qin et al., 2006).

However, previous studies have shown that algorithms making use of quantile normalisation offer the simplest and quickest normalisation methods and also gave the most reproducible results on gene expression and the highest correlation coefficients with Real Time RT-PCR data (Bolstad et al., 2003; Millenaar et al., 2006). Both RMA and GCRMA incorporate the use of quantile normalisation by using data from all arrays to create the same empirical distribution of intensities for each array (Irizarry et al., 2003ab). These methods were used in the current study for normalisation. These two algorithms can be implemented using the **rma** and **gcrma** functions in BioConductor (Gentleman et al., 2004). Their expression measures are based on different background correction methods, but the same quantile normalisation and expression value summarisation using the median polish algorithms (Irizarry et al., 2003a; Irizarry et al., 2003b; Wu and Irizarry, 2004).

Quantile normalisation in RMA and GCRMA functions is followed by a log-transformation step. In this process, the background corrected and quantile

normalised probe values are log transformed usually to a log base 2 (Irizarry et al., 2003b). This stage is necessary to facilitate easy comparison of data of different orders of magnitude.

Summarisation in both RMA and GCRMA results in a set of probe level expression values for each array involved in the gene expression analysis. This involves the use of a robust procedure such as median polish to estimate model parameters for correcting for outlier probes (Irizarry et al., 2003ab).

3.1.7 Differential Expression

The main goal of any microarray data analysis is to detect differentially expressed genes and a number of statistical tests are available to achieve this. For instance, given that this study aims to investigate the effect of acute ethanol exposure on gene expression in *Drosophila melanogaster* exposed to an acute dose of ethanol, the approach will involve detecting differential gene expression between ethanol-treated and control samples in an Affymetrix *Drosophila* array system. To accomplish this, a null hypothesis of no expression level difference between the two sample conditions is proposed. The alternative hypothesis is that there is difference in the level between the two sample conditions. The hypothesis testing can be carried out by performing a statistical test (in this case, t-test) on the expression values of the gene of interest measured in the two conditions. This results in a computed value which can be compared with a threshold t value known as t_α calculated from a t-distribution model and a desired significance level (Tarca et al., 2006). The t-test assesses whether the means of two groups are statistically different from each other. This statistic achieves this goal by examining the differences between the means relative to the spread or variance of the data (Olson, 2006).

Other statistical tests often used in gene expression analysis include the fold change and the ANOVA. The ANOVA test is used if three or more groups are being considered and compared (Nadon and Shoemaker, 2002). This can either be one-way ANOVA if only one factor is being examined or two-way ANOVA when examining two factors (Olson, 2006). Fold change (FC) is the simplest method for identifying differentially expressed genes and is based on the observed ratio (or

average of ratios) between two conditions (Cui and Churchill, 2003). It is the ratio of the measured value for an experimental sample to the value for the control sample. This test is often regarded as an inadequate statistical test because it does not incorporate variance and the differentially expressed genes are not selected based on any significant level of confidence (Miller et al., 2001). Many researchers use FC because it works well for ranking results. This is presumably because all transcripts go through the same processing together, and therefore have similar variances (Allison et al 2005). Both the FC and the t-test statistical criteria can be summarised using an easy-to interpret graph known as **volcano plot** (Figure 3.7). A volcano plot is a device that arranges genes along dimensions of biological and statistical significance (Cui and Churchill, 2003). Thus, it places genes on a two axis coordinate systems. The y-coordinate corresponding to statistical difference is the negative $\log_{10}$ of the p-values for the corresponding statistical differences between the two sample conditions. The x-coordinate, corresponding to biological effects, is the $\log_2$ of the FC between two sample conditions. Genes with statistically significant differential expression (i.e. genes that shows both statistical significance and biological significance) according to the gene-specific *t*-test will lie above an arbitrarily chosen horizontal threshold line (Cui and Churchill, 2003).

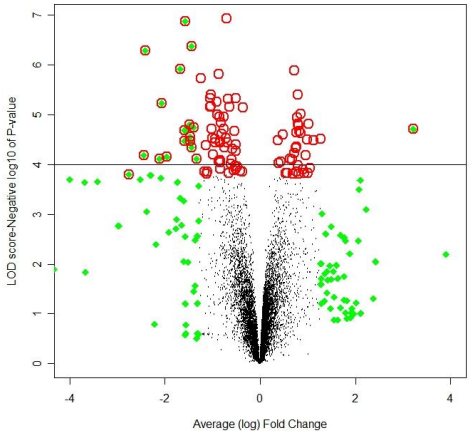


Figure 3.7: Volcano Plot with moderated t-statistics made from the 2 h microarray data used in this study. It shows 100 potentially interesting genes from a biological standpoint.

However, all statistical inferences are associated with a probability of being incorrect (Nadon and Shoemaker, 2002). Methods are therefore needed to minimise inferential errors such as type I error (false-positive error), type II error (false-negative error) and the long range error rate (which defines the expected error rate if experiments and analyses of the type under consideration were repeated an infinite

number of times) (reviewed by Allison et al., 2005). Type I error occurs when the null hypothesis is incorrectly rejected. In a microarray experiment an accumulation of type I errors for each gene can result in a substantial number of false positives (genes incorrectly identified as differentially expressed). Conversely, type II error occurs when the null hypothesis is incorrectly accepted resulting in false negatives: an example is failing to identify genes as differentially expressed when they are.

The percentage of the inferential error discussed above can be determined using *p* values. For example, an error rate of 1% (i.e. p-value of 0.01) means that on average there will be one false positive for every 100 genes identified as differentially expressed. This might be acceptable in an individual test, but in a microarray experiment with very large number of genes, a considerably high number of false positives results may be found. This therefore calls for an adjustment in multiple hypothesis testing (Benjamini and Hochberg, 1995; Dudoit et al., 2003). Accordingly, two methods have been proposed to address the problem of multiple testing:

Family-wise error-rate control (FWER): Using FWER, the probability of finding at least one false positive is minimised (Nadon and Shoemaker, 2002). It is the overall probability that at least one gene is incorrectly identified in the list of differentially expressed genes over a number of statistical tests (Olson, 2006). For instance, if we identified 1000 genes with an adjusted FWER p value of 0.01, then, there is a 1% chance of having one false positive in the list of 1000. The single step Bonferroni correction is the best known method to control the FWER. It defines an effective rate as the standard false positive rate divided by the number of tests conducted (e.g.0.01/1000) (Nadon and Shoemaker, 2002). This means that every individual gene must have a *p* value lower than 0.00001 to be significant. This highly stringent control often results in an increased rate of false negatives results (Nadon and Shoemaker, 2002). Thus, FWER is more appropriate for analyses in which a single positive is unacceptable, such as comparing various drug treatments with a control. Other known methods of FWER include the step down correction method and permutation based one step correction method (Nadon and Shoemaker, 2002).

False-discovery rate control (FDR): The FDR is the expected proportion of false positives among the rejected hypotheses (Olson, 2006). The FDR adjusts the *p* values so that it reflects the frequency of false positives in a list of differentially expressed genes. Thus, if we identified 1000 genes with an adjusted FDR *p* value of 0.01, then there will be an estimated 10 false positives among the 1000 list of genes. A simple procedure for this approach is that proposed by Benjamini and Hochberg, 1995. FDR is less conservative than FWER and is more applicable in screens for candidate genes in which a small proportion of false positives among the discovered genes is acceptable (Olson, 2006). Hence, the FDR approach is used in this study.

3.1.8 Previous analysis

Section 1.6 of Chapter 1 has reviewed some recent and significant studies using microarray experiments with alcohol. Only three of these studies employed *Drosophila* to profile gene expression changes following acute ethanol exposure (Morozova et al., 2006, Morozova et al., 2007 and Urizar et al., 2007). However, these studies differ from the current analysis in the following ways.

- There were differences in experimental designs across the three studies (see section 1.6, Table 1.1 of Chapter 1) and the current study (section 3.2.1). For instance, the Morozova studies profiled gene expression in the whole organism and as such transcriptional changes seen may not be restricted to the *Drosophila* head. Moreover, such analyses involve either the gene expression obtained following a null, single, and double ethanol exposure(s) (Morozova et al., 2006) or the transcriptional changes due to variations across alcohol resistant, alcohol sensitive and control artificially generated fly lines (Morozova et al., 2007). In contrast, the Urizar study profiled gene expression in the *Drosophila* head, however such an analysis involved the use of three different groups of flies treated with different ethanol treatments for fast and chronic ethanol tolerance. Thus these three studies may not account for the whole time-dependent transcriptional changes that were discovered in the current study.
- Different statistical tests were used to select list of differentially expressed genes. Morozova employed a combination of MAS 5.0 with either one-way

fixed effect ANOVAs (Morozova et al., 2006) or two-way fixed effect ANOVAs (Morozova et al., 2007) of the *Signal* metric using the general linear model (GLM) procedure in SAS statistical software. Urizar made use of dChip program with one-way ANOVA for differential analysis. The current work has used RMA and GCRMA normalising methods together with a modified form of t-statistics (or one-way ANOVA for trends analysis) implemented in linear modelling for microarray analysis (LIMMA) within the BioConductor statistical software.

3.2 Materials and Methods

3.2.1 Microarrays Data

The microarray data used in this study was generated by Hancock, 2005. In brief, 100, 2-5 day old male flies from an isogenic Oregon R (OrR) strain were anaesthetised using ice and placed into acrylic exposure boxes measuring 6cm x 6cm x 1.5cm internally with a fully removable lid and separate input/output tubes. After 30 min recovery and acclimatisation period, they were then exposed to 15 minutes of vapour produced by bubbling air through 300ml 98% EtOH (45°C) at 0.4l/min and 100ml water (unheated) at 0.2l/min and in control samples, 300ml water (45°C) was used in place of EtOH. After exposure, the flies were placed in 25ml falcon tubes with cotton wool bungs for a defined recovery period of 0, 0.25, 0.5, 1, 2, 3, and 4 h. Control flies were kept after exposure in the same environment as the experimental flies and culled at the same recovery period. Total RNA was extracted using Trizol from fly head of the samples and used to generate biotin-labelled cRNA for hybridisation to GeneChip array. Chips were hybridized at the Institute of Child Health (for 0.5, 2, and 4 h time points of both the ethanol and control cases (Sussex Chips) or sent to the Glasgow *Drosophila* Affymetrix facility (for 0.25, 1 and 3 h time points of both the ethanol and control cases (Glasgow Chips). After extensive washing, the microarrays were dried, scanned and quantitated for signal intensities stored in .CEL files.

In the current work, all pre-processing and differential analysis of the microarray data were conducted using BioConductor version 2.1 within R software (version 2.6.0) installed under Windows Vista™. BioConductor (Gentleman et al., 2004) is a free and open development software specifically designed for the analysis and comprehension of genomic data such as microarray data. It is based primarily on the statistical R programming language but contains contributions on other programming languages (Gentleman et al., 2004).

3.2.2 Data Quality

Data quality checks on the microarray data were carried out. These involve assessing the chips for their quality using six different BioConductor (www.bioconductor.org) quality assessment tools. Seven time points (sub-divided

into Sussex and Glasgow groups) with 8 common reference controls (sub-divided into 5 Sussex controls and 3 Glasgow controls) and totalling 30 microarrays were used in this study (See section 3.2.1 and Table 3.1). The 2 h time point (from Sussex group) with its common reference controls totalling nine arrays were used to determine differential gene expression between ethanol-treated and control flies (Figure 3.5). These 2 h dataset was chosen because preliminary analysis showed that it contained the highest number of gene expressions compared to other data sets (data not shown). In addition, this dataset has the highest number of possible replicates (4 ethanol-treated and 5 control samples). The use of replicates aids in minimising the effects of chance variation and allows the extent of experimental variation to be estimated (Olson, 2006). All seven time points (both Sussex and Glasgow groups) were used to test for time-dependent effects in acute ethanol modulation of transcriptional changes in *Drosophila* head (Figure 3.5).

Time (h)	**No of EtOH chips**	**No of H_2O chips**	**Group**
0	3	$5^{!}$	Sussex
0.25	3	$3^{\dagger}$	Glasgow
0.5	3	$5^{!}$	Sussex
1	3	$3^{\dagger}$	Glasgow
2	4	$5^{!}$	Sussex
3	3	$3^{\dagger}$	Glasgow
4	3	$5^{!}$	Sussex
Totals	**22**	**8**	

Table 3.1: A summarised information on all the *Drosophila* Affymetrix chips used in this study. ! and † denote the two common reference controls specific for Sussex and Glasgow chips respectively.

3.2.3 Normalisation

Gene expression data were normalised for all the nine GeneChips of the 2hr time point by computing the Robust Multichip Average (RMA) (Guantier et al., 2004) and Guanine Cytosine-RMA(GC-RMA) incorporating the sequence model (i.e. GC content) of each probe (Wu et al., 2004). After normalisation, a fast numeric model parameter estimating technique known as median polish (Irizarry et al., 2003a) was then applied to compute one expression measure for all the probe values resulting in a $\log_2$ transformed RMA and GCRMA expression values.

For the time-dependent (time series data) analysis, the gene expression data was normalised for each gene/time period by using both the Sussex and the Glasgow controls to calibrate all the time groups all on the same scale using quantile normalisation in RMA. It should be noted that only the RMA algorithm was used for normalisation of these time series data because it showed better normalisation results on the pooled data than GCRMA (Figure 3.13 a and b; Appendix A.1.). The normalised measurements at each time point were then assessed using various diagnostics tools and the data were treated as a common dataset since we had reference controls to factor out the laboratory effect. The main problem with this type of design is that one may not be able to distinguish which of the factors (i.e. time or laboratory effect) account for any significant difference in gene expression. However, since there were common reference controls in both laboratories, the control data then provides an unbiased estimate of the lab effect for each gene. By normalising, one can factor out the laboratory effect and perform an unbiased comparison of the different time groups to each other. After normalisation, the normalised time series data were fitted to a global model of expression and probe affinities to compute expression values as previously described.

3.2.4 Differential Expression

Differential gene expression between ethanol-treated and humidified water vapour-treated flies was assessed using empirical Bayes approach in LIMMA method (Smyth, 2004). Empirical Bayesian procedures in LIMMA analysis allows the selection of probesets differentially expressed between ethanol and control regimes in each algorithm (Smyth et al., 2004). The experimental design of including only 3 to 4 biological (i.e. ethanol) replicates most likely limited detection of very low expression changes, particularly of low-abundance genes. However, it was found that LIMMA method together with the choice of our algorithms is particularly useful for studies having limited numbers of Affymetrix microarrays. This is because the empirical Bayes analysis implemented in LIMMA allows for the analysis of gene expression microarray data involving small replicates with increased statistical power (Smyth 2004). The LIMMA analysis requires the definition of a design and contrast matrix to fit a linear model by least squares (Smyth et al., 2006). The design matrix represents the RNA target hybridised on the GeneChip while the contrast matrix enables the coefficient in the design matrix to

be combined into specific comparisons (Smyth et al., 2006, Smyth 2005). A design matrix was created that included separate coefficients for the control and ethanol treated GeneChips and then the difference was extracted as a contrast (Figures 3.8 and 3.9).

```
T2<- c ("W1 0h.cel","W2 0.5h.cel","W3 2h.cel","W4 2h.cel","W5 4h.cel",
"E2h 1.cel","E2h 2.cel","E2h 3.cel","E2h 4.cel")
raw_Data<- ReadAffy (filenames=T2)
eset <- gcrma (raw_Data)
design <- cbind (WAT=c(1,1,1,1,1,0,0,0,0), ETH2=c(0,0,0,0,0,1,1,1,1))
fit <- lmFit (eset, design)
cont.matrix <- makeContrasts (WATvsEHT=ETH2-WAT, levels=design)
fit2 <- contrasts.fit (fit, cont.matrix)
fit3 <- eBayes (fit2)
tab <- topTable (fit3, adjust="fdr", sort.by="p", number=14010)
```

Figure 3.8. R code for the 2 h (T2) differential gene expression analysis showing the design and contrast matrix defined within the LIMMA statistical package. Chips beginning with "W" denote H_20 treated (WAT) control samples while those beginning with "E" denote EtOH treated (ETH2) samples.

```
TS <- c ("E0h 1.cel","E0h 2.cel","E0h 3.cel", "E0.25h 1.cel","E0.25h 2.cel","E0.25h 3.cel", "E0.5h
1.cel","E0.5h 2.cel","E0.5h 3.cel","E1h 1.cel","E1h 2.cel","E1h 3.cel", "E2h 1.cel",
"E2h 2.cel","E2h 3.cel","E2h 4.cel", "E3h 1.cel","E3h 2.cel","E3h 3.cel", "E4h 1.cel",
"E4h 2.cel","E4h 3.cel","W1 0h.cel","W2 0.5h.cel","W3 2h.cel","W4 2h.cel","W5 4h.cel",
"W6 0.25h.cel","W7 1h.cel","W8 3h.cel")
raw_Data <- ReadAffy (filenames=TS)
eset <- rma (raw_Data)
design<-model.matrix(~1+factor(c(1,1,1,2,2,2,3,3,3,4,4,4,5,5,5,5,6,6,6,7,7,7,8,8,8,8,8,8,8,8)))
colnames(design)<-c("E0Hrs","E0.25Hrs","E0.5Hrs","E1Hrs","E2Hrs","E3Hrs","E4Hrs","WsHrs")
fit <-lmFit (eset, design)
contrast.matrix < - makeContrasts (E0Hrs-WsHrs, E0.25Hrs-WsHrs, E0.5Hrs-WsHrs,
E1Hrs-WsHrs, E2Hrs-WsHrs, E3Hrs-WsHrs, E4Hrs-WsHrs, levels=design)
fit2 <- contrasts.fit (fit, cont.matrix)
fit3 <- eBayes (fit2)
tab <- topTable (fit3, adjust="fdr", sort.by="p", number=14010)
```

Figure 3.9. R code for the time series (TS) differential gene expression analysis showing the design and contrast matrix defined within the LIMMA statistical package. Chips beginning with "W" denote H_20 treated control samples at various time points while those beginning with "E" denote EtOH treated samples at various time points. WsHrs denote H_20 treated common reference control for all the time points.

(a) 2 hr time point: The normalised expression values from the 2 h data were analysed using R/moderated t-statistics by fitting a linear modelling contrast for the two conditions of ethanol-treatment and control (Figure 3.8) (Smyth 2004):

$$ß_g = C^T \alpha_g \quad (1)$$

where ß denotes a vector of contrasts for gene g, C is the contrasts matrix, and α_g is a vector of coefficient (i.e. estimated log fold changes) obtained from a linear model fit.

For estimation of differential gene expression from the 2 h Sussex data, the LIMMA method controlling FDR was used to compute two lists of differentially expressed genes at two different threshold levels of stringency:

a) Stringent analysis: The list of genes from this analysis was computed using overlaps of genes across RMA and GCRMA that met a FDR cut-off of 5 %. This list is referred to P-0.05 in this thesis.
b) Non-stringent analysis: The list of genes from this analysis was computed using overlaps of genes across RMA and GCRMA that met a FDR cut-off of 10 %. This list is referred to P-0.1 in this thesis.

Genes that showed significant differential expression from the P-0.05 were further analyzed using linear regression to evaluate the concordance of fold changes (FC) obtained from the two alternative algorithms (Figure 3.14). This list of genes was also used to query the FlyBase data base (http://www.flybase.bio.Indiana.edu) for their individual GO categories (The Gene Ontology Consortium, 2000). Finally, the list allowed candidate genes to be identified and taken to the laboratory for validation. On the other hand, the P-0.1 list was used in functional analyses (e.g. computational analysis of pathways and networks). The somewhat liberal FDR approach in LIMMA analyses for the P-0.1 increased our ability to populate functional clusters of genes in subsequent computational studies.

(b) Time series: In addition to detecting differential gene expression between the control and ethanol-treated samples at a single time point (2 h), it is also of interest

to detect trends in gene expression across time. This question was addressed through an ANOVA analysis in LIMMA (Smyth, 2004) using a model fit:

$$\mathbf{E_{ijk}=T_i+S_j+(TS)_{ij}+\varepsilon_{ijk}} \quad \textbf{(2)}$$

where E_{ijk} represents the measured gene expression for treatment i, time j, and biological replicate k, with $1 \leq i \leq 2$, $1 \leq j \leq 7$, and $1 \leq k \leq 8$. The E_{ijk} is assumed to be the results of the added effects of factors time (T) and treatment (S) over a time point j and treatment i, $(TS)_{ij}$ accounts for the interaction between treatment and time (Davletova et al., 2004).

Using this model, seven contrasts (at 7 time points of 0, 0.25, 0.5, 1, 2, 3, and 4 h) based on differences between ethanol and common reference controls were estimated for the study of time-dependent changes in transcriptional profiles of *Drosophila* head exposed to acute ethanol (Figure 3.9). The moderated F-statistics was employed to combine the t-statistics for all the contrasts into an overall test of significance for each probe (i.e. gene) (Smyth, 2004). This F-statistic tests whether any of the contrasts are non-zero (null hypothesis) for each gene i.e. whether the gene is differentially expressed on any contrast. This F-statistic is similar to the ordinary F-statistic from analysis of variance except that the denominator of mean squares are moderated across genes (Smyth, 2004). A multiple testing correction (Benjamini and Hochberg, 1995) was applied to adjust the *p*-values of the F-statistic.

3.2.5 Functional Clusters and Pathway Analysis

The functional classification of differentially expressed genes was carried out using the P-0.1 list of genes. This is because highly stringent filtering such as that involving P-0.05 may have removed differential expressed genes forming part of a cluster (s) thereby leading to loss of functional gene clusters revealing the biology underlying alcoholism.

(a) DAVID (Database for Annotation, Visualisation and Integrated Discovery):
Several programs have been developed for functional annotations of genes derived from microarray data and these include FatiGO (Al-Shahrour et al., 2004), GoMiner (Zeeberg et al., 2003), MAPPFinder (Doniger et al., 2003), and MatchMiner (Bussey et al., 2003). However, while these programs share many overlapping and

related functionalities, DAVID was chosen for the current work because of its robustness which stems from its combination of features within a single platform (Dennis et al., 2003). Indeed, DAVID is a program of choice for many researchers working with genomic data, with over 1,000 papers citing DAVID from many research institutes world wide (http://david.abcc.ncifcrf.gov/). For instance, DAVID has been used to identify gene networks that may contribute to the genetic susceptibility of autism spectrum disorders (ADS) – a childhood neurodevelop-mental disorders with complex genetic origins (Glessner et al., 2009) and also in establishing the enrichment of genes mediating immunogenicity of the yellow fever vaccine in humans (Querec et al., 2009).

DAVID identifies general categories such as GO terms, keywords, Smart name, Interpro name etc present in a list of target genes (Dennis et al, 2003, Hosack et al 2003). DAVID gene functional classification and the clustering tools provide a module-centric approach for functional analysis of gene lists (Huang et al 2007). The up-and down-regulated gene sets from the P-0.1 list were separately annotated and grouped into functional annotation and enrichment terms using the DAVID software (Dennis et al., 2003). In this way, DAVID measures gene-gene and term-term similarities based on the assumption that genes that share global functional annotation profiles and terms that share global gene profiles are functionally related to each other respectively (Huang et al 2007). DAVID scores the enrichment of each GO term based on kappa statistics (Huang et al 2007). In addition, DAVID uses a fuzzy heuristic partitioning method to group related genes or terms into functional themes (biological modules) based on the similarity distances measure ((Huang et al 2007, Hosack et al 2003). A kappa similarity threshold value of ≥ 0.35 and a multiple linkage threshold value of ≥ 0.50 (all default settings) were applied for the functional analysis. The count i.e. group size was limited to a minimum number of two.

(b) KEGG spider: Several programs have been developed for representing and interpreting genes derived from microarray data in a global metabolic network and these include GENECODIS (Carmona-Saez et al., 2007), Pathway Miner (Pandey et al., 2004), and KEGG atlas (Okuda et al., 2008). The choice of KEGG spider in the current work is because it takes into account the density of the metabolic

networks for estimating the statistical significance of the model quality (Antonov et al., 2008). KEGG spider has been used to reveal changes in the network of genes contributing to cell motility in mouse embryo fibroblast lacking stathmin, a microtubule regulatory protein (Ringhoff and Cassimeris, 2009).

In the current work, KEGG spider (Antonov et al., 2008) was used to identify metabolic pathways and networks enriched from the acute ethanol regulation of gene expression in *Drosophila* head using the P-0.1 list of genes. For the pathway analysis, the up-and down-regulated gene sets were separately used (Figure 3.15a). In contrast, for the network analysis, the total differentially regulated genes were used (Figure 3.16). KEGG spider identifies enriched metabolic pathways present in the list of target genes. It also implements a global metabolic network framework for the interpretation of the gene lists. In this way, it translates the gene lists into network models using a robust Monte Carlo simulation statistical procedure to estimate the significance of the models (Antonov et al, 2008). This significance (*p* value) score represents a probability to infer the same or bigger size models from a randomly generated gene list of size equal to the size of the input list. KEGG spider computes the minimal distance between any two genes as a minimal number of steps required to get from one gene to another (Antonov et al, 2008). A random network value setting of 200 was used to generate the background distribution and estimate the statistical significance of the inferred model based on the distribution of the model size for a random gene list. The graphs of the metabolic networks were created using Medusa, a simple application for visualising and manipulating graphs of interaction (Hooper and Bork, 2005).

3.2.6 Cluster Analysis

Many methods exist to cluster time series data. These include hierarchical clustering (HC), K-means clustering and self-organising maps (SOM) methods. HC is often used to identify gene expression patterns in a large microarray data set (Olson, 2006). HC builds clusters of genes with similar expression profiles and uses a dendogram that assembles all the elements of these profiles (matrix) into a single tree (Olson, 2006). Several methods can be employed to build this tree including single-linkage clustering, complete-linkage clustering and average-linkage clustering.

The hierarchical clustering method was pioneered by the Brown and Botstein lab and they make use of TREEVIEW (Brown and Botstein, 1999) which is now one of the most widely used tools in functional genomics. K-means clustering method is a partitioning method often used to separate data into discrete clusters (Olson, 2006). K-means allows the user to specify the number of clusters to be identified. This method of clustering is also implemented in the STEM algorithm used in this study (Ernst and Bar-Joseph, 2006). SOM clustering technique is based on a neural network system designed for better exploratory data analysis. SOM is particularly well suited for identifying a small number of prominent classes in a data set (Tamayo et al., 1999) and like K-means clustering, the user specifies the number of clusters to be identified. A Self-organising map then finds an optimal set of centroid around which the data points appear to aggregate. It then partitions the data set, with each centroid defining a cluster consisting of the data points nearest to it (Golub et al., 1999). This method has been shown to be effective at automatically discovering two types of leukaemia in one of the first publications that showed how microarray analysis can assist in difficult clinical diagnosis (Golub et al., 1999).

TM4 software (Saeed et al., 2003) contains the functionalities for the implementation of all of the above clustering methods and was used to depict the expression profiles of the time-dependent list of ethanol-regulated genes (FDR <0.01) in heat map (Figure 3.17 a).

The Short Time-series Expression Miner (STEM) software (Ernst and Bar-Joseph, 2006) contains the functionalities for K-means and STEM clustering and was used to cluster the time-dependent expression changes represented on the heat map into different gene classes. STEM has been successfully used by many excellent studies in clustering expression data most especially to depict early, medium and late regulated genes in various experimental preparations (Baker and Russel, 2009; Capra et al., 2008; Martinez et al., 2007; Yang et al., 2009). In the current study, STEM was used to identify biologically relevant clusters culminating in putative and prominent classes of genes in time series that showed differential expression in response to acute ethanol. The list of genes obtained from a time-dependent analysis was subjected to cluster analysis in STEM stand alone application

software. The choice of STEM in the current study is because it is particularly well suited to the task of clustering a short time series (3-8 time points) microarray gene expression data (Ernst and Bar-Joseph, 2006). This algorithm starts by selecting a set of potential expression profiles. These sets of profiles cover the entire space of possible expression profiles that can be generated by the genes in the experiment and each represents a unique temporal expression pattern (Ernst, et al., 2005). Thus, for a short time series data like the one used in the current study, a relatively small set of profiles can be defined and represented in this case.

Upon the input of the gene list, the STEM algorithm first selects a set of distinct and representative temporal expression profiles, called model profiles, independently of the data and then assigns each gene in the list to them based on how closely matched to the model profiles are the gene's expression matrices (profiles) as determined by the correlation coefficient. The algorithm then determines which of these profiles have statistically significant higher number of genes assigned using a permutation test. To define a set of model profiles the user defines a parameter that controls the amount of change a gene can exhibit between successive time points (Ernst et al., 2005). The significant profiles can either be analysed independently or grouped into larger clusters (based on noise estimates from the data). A non-correction statistical method with a level of significance (p value) of 0.1 and minimum absolute expression change of 0 were defined upon the input of the gene list for clustering in this work. A zero minimum absolute expression change ensured that no single gene was filtered out during the analysis while the non-correction method with a p value of 0.1 helped to increase the statistical significance of the model profiles without altering the arrangement of genes on these profiles. All other parameters used were default.

3.3 Results

3.3.1 Data Quality

Quality control checks on all the nine Gene Chips of the 2 h time point Sussex data revealed that all the chips were found to be of comparable and good quality. A series of graphical tools and statistical summaries used in carrying out various diagnostics measures from the well known summary plots such as boxplots (Figure 3.9), histograms (Appendix A.2) and MA plots (Appendix A.3) to the more advanced procedures involving fitting the probe-level model using Relative Log Expression (RLE), Normalised Standard Errors (NUSE) plots (Figures 3.11) and summarising residuals and weights (data not included) from them had helped to inform the decisions as to why all these nine arrays were included in the subsequent analysis.

The graphs in both the boxplots (Figure 3.10) and the density plots (Appendix A.2) clearly show the variation (differences in spread and position) in probe intensities across all the Gene Chips. These variations do not indicate any potential problems with experimental conditions but only suggesting the need to normalise the data.

Using NUSE and RLE for quality assessment is based on the assumption that the majority of genes are not differentially expressed. In this research, it is reasonable to assume that the majority of the genes are not changing in expression between the 2 experimental conditions of ethanol treated and control samples. The majority of these non-differential genes are then shown on the NUSE and the RLE plot by the boxes. NUSE is used to identify any arrays which have elevated standard errors (SE) relative to other arrays in the dataset (Bolstad et al 2005). Thus a good quality array has a median NUSE around 1 and small inter-quartile range (IQR). Conversely, an array with low quality might be indicated by a box that is significantly elevated or more spread out relative to the other arrays (high IQR) in the plot and with high values of median NUSE (Bolstad et al 2005). All the arrays in the NUSE plot shown in Figure 3.10 centre around 1 and therefore present no quality problems. RLE values can also be used to define a good quality array. A good quality array has a median RLE around 0 and a small IQR while an array with quality problems may be seen in a box that has relatively greater spread or that

is not centred around 0 (Bolstad et al., 2005). As can be visualised from the RLE plot in Figure 3.11, all the arrays centred around 0 and with approximately equal sizes. Thus, these plots show no quality problems in our dataset.

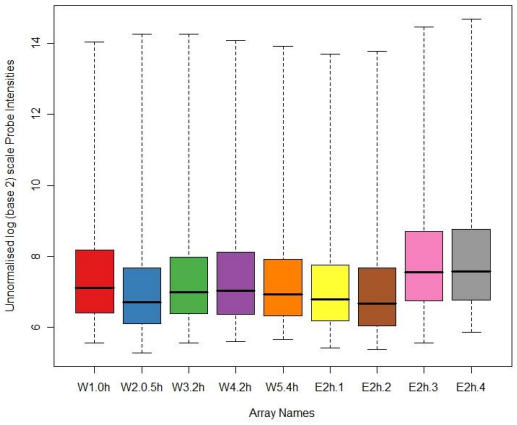


Figure 3.10: Boxplots of nine arrays of probe-level *Drosophila* head data. Each box in the boxplots representing inter-quartile range overlaps each other to a large extent. This suggests good chips quality. The first five boxplots correspond to the control condition and last four the treatment conditions of the nine arrays.

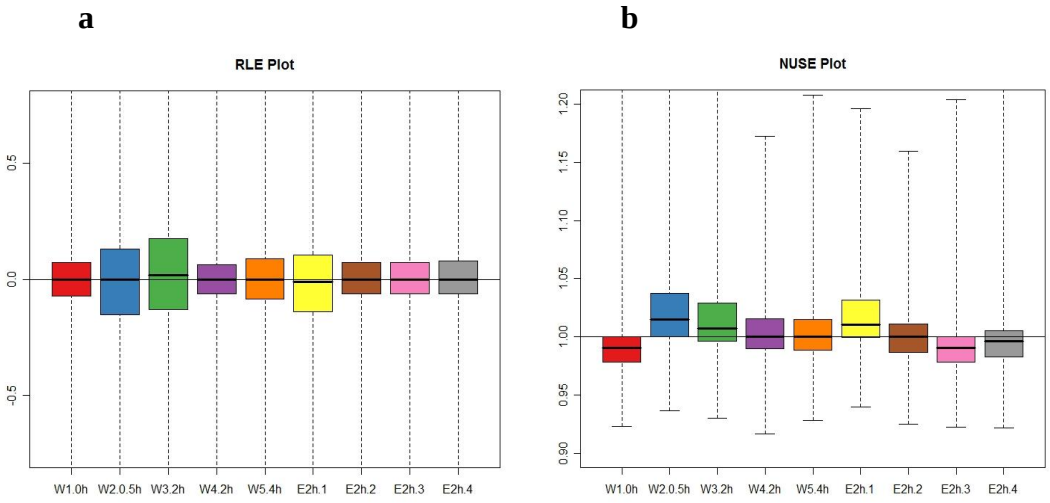


Figure 3.11: (a) shows the NUSE while (b) shows the RLE for the 2 h data used in this study

3.3.2 Normalisation

Graphical verifications of the RMA and GC-RMA normalised expression data of all these nine arrays using boxplots (Figure 3.12), histogram plots (Appendix A.2) showed that all the arrays are aligned and equally distributed. Thus, the variations found within the probe-level data (unnormalised nine GeneChips in Figure 3.10) which may obscure interesting biological differences between ethanol and control *Drosophila* head samples have been accounted for by the normalisation process.

RMA
a

GCRMA
b

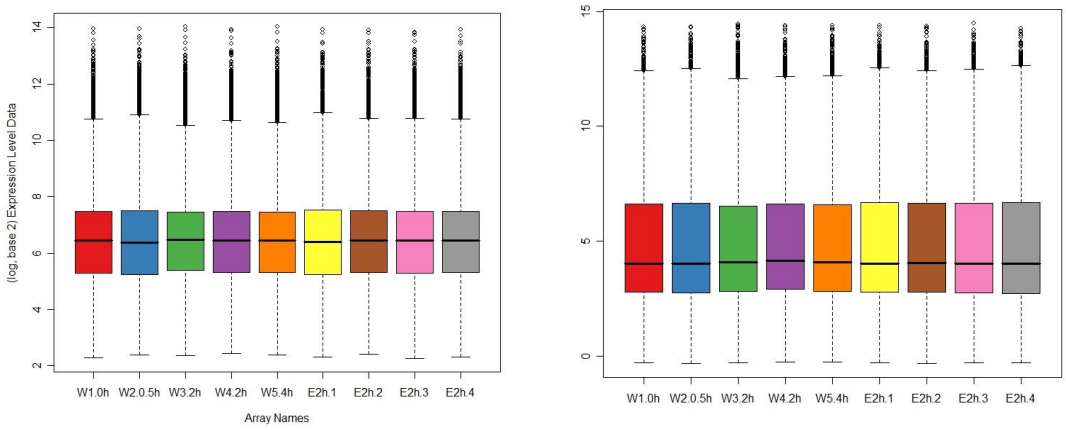


Figure 3.12: shows (a) and (b) box plots and of the nine arrays obtained using the two different algorithms. One can see that these arrays are aligned together and equally distributed.

Quality checks were also carried out on the whole time series data to assess the effect of pooling on both the Sussex and Glasgow data sets. This is to ensure that treating the two data sets as a common dataset is reliable. In addition, this will ensure that comparisons that are biologically relevant to this present investigation are being made. The results, as shown below (Figure 3.13), indicate that normalisation using the RMA method is effective to account for the variations in the pooled array data.

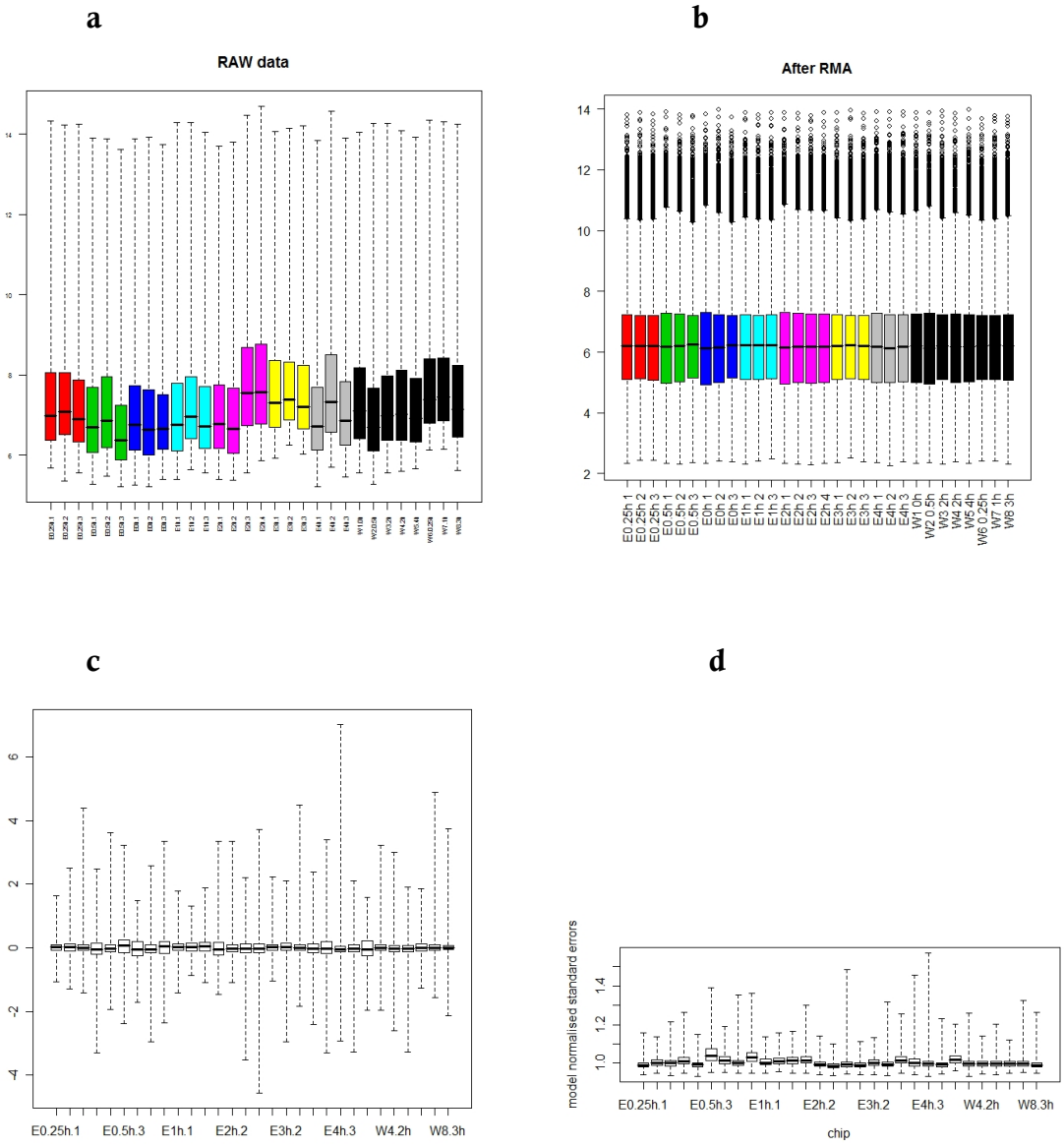


Figure 3.13: The boxplots (a) of the raw time course data before normalisation (b) of the normalised time course data using RMA normalising procedure (c) of expression measure relative to pseudomedian chip showing all the pooled arrays centred around 0 and indicating no quality problems in our pooled data (d) by chip of standard errors of expression values, standardised to median 1 showing that the pooled chips have comparable standard errors to one another.

3.3.3 Differential Expression

As described in section 3.2.4 (a) of Materials and Methods, statistical analysis of the 2 h data was performed on *Drosophila* head from 4 ethanol-treated and 5 control samples to identify alteration in gene transcription associated with ethanol response. This analysis culminated into two different list of genes P-0.05 and P-0.1 representing stringent and non-stringent analysis results respectively. Section 3.1.4, Figure 3.5a shows further analysis work flow carried out on these two separate lists of genes.

(a) Stringent analysis- P 0.05

In the stringent analysis, 155 genes met the criteria [excluding genes not available (i.e. Affy IDs (probe sets) where no corresponding gene symbol were available at the time of annotation) and duplicate genes (i.e. gene corresponding to two different Affy IDs on the array)]. A heat map was created showing the relative expression levels of these 155 genes in ethanol-treated and control samples (data not shown) of which 101 were transcriptionally upregulated (Table 3.2) and 54 were downregulated (Table 3.2). Genes are clustered by their relative expression levels over the 9 samples (5 water and 4 EtOH treated) with hierarchical clustering showing that both the ethanol-treated and normal *Drosophila* head display a complete different pattern of expression (Appendix A.4). Further, a linear regression analysis of fold differences between RMA and GC-RMA for the evaluation of the level of their fold change concordance using a pair-wise up-and downregulated gene sets showed a good fold change correlation between the fold results from these two alternative algorithms (Figure 3.14). Genes were later annotated individually for their biological processes, molecular functions and cellular components as defined by the Gene Ontology (GO) Consortium (The Gene Ontology Consortium, 2000) using *Drosophila* database (http://www.flybase.org). This database also allows orthologous gene to be identified.

As shown in Tables 3.2, the upregulated gene with the largest FC after acute ethanol administration in *Drosophila* head was *heat shock protein 26*, *Hsp26* (FC=26.54), followed by another heat shock gene, *Hsp23* (FC=12.83). These two genes are both involved in stress responses. The upregulated gene with the smallest

FC was *strawberry notch*, *sno* (FC=1.31) followed by *highwire*, *Hiw* (FC=1.38), which has both human and mouse orthologues. These genes are involved in nucleic acid binding and cellular protein ubiquitination respectively. The most significant downregulated transcript (Table 3.3) was *CG11909* (FC=9.29) involved in glucose metabolism. This was followed by *CG18302* gene involved in lipid metabolism (FC=4.69). The downregulated gene with the least FC was *CG2233* (FC=1.28) followed by *CG9928* (FC=1.28). These two genes encode proteins of unknown functions. In general, genes with general stress response functions and genes involved in several signaling cascades are upregulated while most of the genes involved in general metabolism are downregulated. This analysis also revealed that 54 of the upregulated genes have both mouse and human orthologues with 2 genes having only human orthologues (Table 3.2) while 17 of the downregulated genes have both human and mouse orthologues (Table 3.3).

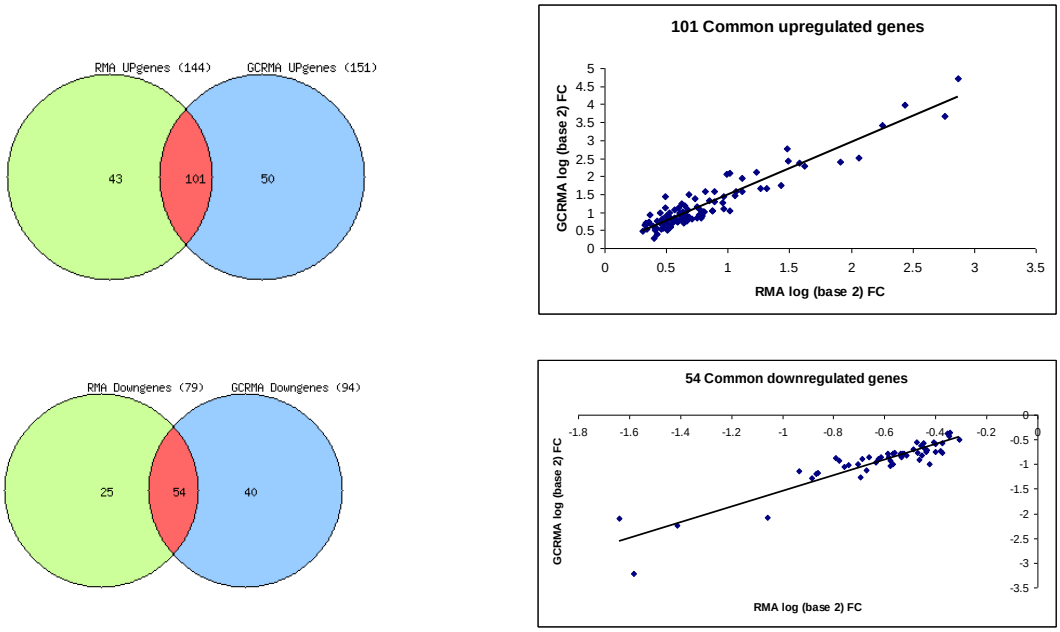


Figure 3.14: Correlation of fold change between alternative normalisations.

(a) Upper pane, RMA Upregulated genes versus GCRMA upregulated genes. (b) Lower pane, RMA downregulated genes versus GCRMA downregulated genes. The solid lines represent a linear regression fit. The overlapping gene lists between the alternative algorithms are represented in the venn diagrams. Linear fit: RMA upregulated genes versus GCRMA upregulated genes, Y=1.4594X + 0.0237, R^2 =0.897; RMA downregulated genes versus GCRMA downregulated genes, Y=1.584X + 0.0436, R^2 =0.8464. The common genes were selected based on Benjamini and Hochberg's false discovery rate method (fdr ≤ 0.05). The Adjusted *P* values of their gene expression changes on the array are shown in Tables 3.2 and 3.3.

Having identified a variety of potential candidate genes from this analysis, the next stage is to carry out biological validation on these genes. It should first be noted that a reasonable number of genes earlier reported to be implicated in ethanol response have been identified in this study (see Table 7.1 in Chapter 7 and Appendix B.3) and thus offer an excellent validation for the candidate ethanol-regulated genes discovered in this study. However, for the purpose of validating the microarray experiments, some filtering criteria were applied to select around seven putative ethanol-regulated genes offering interesting possibilities for future work.

First, the 7 candidate genes for validation was selected from the list of genes obtained from the 2 h microarray data at a FDR (p<0.05) i.e. P-0.05 list of genes. The 7 candidate genes were chosen from this 2 h data because the genes had passed through stringent filtering criteria. Second, mutations to these genes were known to be present while viable alleles of the fly stocks carrying these mutations were sought and confirmed to be available for testing. Thus, all the seven genes were selected based on a method driven by functional hypotheses, availability of stocks and suitability for behavioural genetics testing. These genes are *ana*, *Axn*, *hiw*, *hop*, *hsp26*, *hsp83* and *mbf1*. Descriptions of these genes were covered in Chapter 1 section 1.10. Finally, validation analyses on these seven genes were carried out in Chapter 4.

Upregulated Gene Expression in *Drosophila* Head after Acute Ethanol Exposure

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P*.Value**	**FC**	**Adjusted *P*.Value**
39075	*Hsp26*	*Heat shock protein 26*	protein binding	determination of adult life span; response to heat; defense response; protein folding	-	7.29	0.0082	26.54	0.0431
39077	*Hsp23*	*Heat shock protein 23*	actin binding	response to heat; defense response; protein folding	-	6.77	0.0085	12.85	0.0248
43496	*Obp99d*	*Odorant-binding protein 99d*	odorant binding	autophagic cell death; salivary gland cell autophagic cell death; transport	-	4.77	0.0070	10.59	0.0246
48335	*GstD2*	*Glutathione S transferase D2*	glutathione transferase activity; glutathione peroxidase activity	Defense response; response to toxin	-	2.79	0.0216	6.75	0.0222
38389	*Hsp83*	*Heat shock protein 83 (MH)**	ATPase activity, coupled; ATP binding; unfolded protein binding	anatomical structure development; primary metabolic process; organelle organization and biogenesis; macromolecule metabolic process; defense response; transmembrane receptor protein tyrosine kinase signaling pathway; gamete generation; anterior/posterior axis specification; cellular macromolecule metabolic process; response to stress; cell cycle; sleep (cycadian rhythm regulation); actin filament-based process	Centrosome; cytoplasm	4.18	0.0085	5.73	0.0236
31461	*CG15784*	*CG15784*	Unknown	unknown	unknown	2.80	0.0047	5.44	0.0140
37106	*GstE1*	*Glutathione S transferase E1*	glutathione transferase activity	response to oxidative stress; defense response; response to toxin	-	3.77	0.0006	5.32	0.0018
35707	*CG2065*	*CG2065*	oxidoreductase activity	metabolic process	-	2.98	0.0253	5.19	0.0497
37112	*GstE7*	*Glutathione S transferase E7*	glutathione transferase activity	defense response; oxygen and reactive oxygen species metabolic process; response to toxin	-	3.08	0.0068	4.91	0.0222
32384	*CG32602*	*CG32602*	receptor activity	cell adhesion; defense response	-	2.34	0.0068	4.33	0.0155
37113	*GstE8*	*Glutathione S transferase E8*	glutathione transferase activity	defense response; oxygen and reactive oxygen species metabolic process; response to toxin	unknown	2.02	0.0226	4.23	0.0230
43601	*CG9733*	*CG9733*	trypsin activity; monophenol monooxygenase activator activity	proteolysis; defense response	-	1.98	0.0070	4.18	0.0052
37578	*CG4269*	*CG4269*	Unknown	unknown	unknown	2.17	0.0052	3.89	0.0148
35687	*CG1600*	*CG1600*	zinc ion binding	unknown	unknown	2.69	0.0114	3.34	0.0246
39998	*CG5290*	*CG5290 (MH)**	Unknown	unknown	unknown	2.40	0.0127	3.20	0.0364
33202	*Hop*	*Hsp70/Hsp90 organizing protein homolog (MH)**	unfolded protein binding	protein folding; defense response; protein complex assembly; response to stress	-	2.48	0.0041	3.19	0.0030

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
31838	*CG7033*	*CG7033 (MH)**	ATPase activity, coupled; ATP binding; ATP-dependent helicase activity; nucleic acid binding; unfolded protein binding	protein folding	chaperonin-containing T-complex; lipid particle	1.76	0.0080	3.02	0.0389
37770	*CG4797*	*CG4797*	glucose transmembrane transporter activity	carbohydrate metabolic process; carbohydrate transport	integral to membrane	1.85	0.0137	3.00	0.0094
50446	*CG16978*	*CG16978*	Unknown	unknown	unknown	2.17	0.0108	2.99	0.0082
36308	*Cct5*	*T-complex chaperonin 5 (MH)**	ATP binding ATPase activity, coupled unfolded protein binding	protein folding	chaperonin-containing T-complex	2.09	0.0033	2.98	0.0009
35621	*Tsp42Ek*	*Tetraspanin 42Ek*	receptor signaling protein activity	ectoderm development; nervous system development; transmission of nerve impulse	integral to membrane	1.61	0.0041	2.80	0.0079
35426	*CG1416*	*CG1416 (MH)**	Unknown	unknown	unknown	2.28	0.0085	2.78	0.0094
37744	*l(2)efl*	*lethal (2) essential for life (MH)**	Unknown	embryonic development; response to heat; defense response; protein folding; response to stress	-	2.08	0.0068	2.77	0.0094
31565	*Top3beta*	*Topoisomerase 3β (MH)**	DNA topoisomerase activity; endodeoxyribonuclease activity; DNA topoisomerase type I activity; nucleic acid binding; ATP binding; aminoacyl-tRNA ligase activity	DNA catabolic process, endonucleolytic; DNA modification; DNA topological change; DNA unwinding during replication; tRNA aminoacylation for protein translation	chromosome	1.40	0.0161	2.72	0.0106
33265	*kraken*	*kraken (MH)**	serine hydrolase activity	digestion; response to toxin; aromatic compound metabolic process	cellular_component	1.95	0.0015	2.71	0.0018
35882	*CG8258*	*CG8258 (MH)**	ATPase activity, coupled; ATP binding; unfolded protein binding	protein folding	chaperonin-containing T-complex	1.66	0.0110	2.63	0.0082
39557	*Hsc70Cb*	*Hsc70Cb (MH)**	chaperone binding; ATP binding	protein folding; defense response; response to stress	-	1.80	0.0249	2.51	0.0154
32992	*CG12703*	*CG12703 (MH)**	ATP binding; ATPase activity, coupled to-transmembrane movement of –substances; transporter activity	lipid metabolic process lipid transport peroxisome organization and -biogenesis	ATP-binding cassette (ABC)- transporter complex integral to peroxisomal membrane	1.85	0.0080	2.44	0.0268
34176	*fu12*	*fu12*	1-acylglycerol-3-phosphate O-acyltransferase activity	phospholipid metabolic process; phospholipid biosynthetic process	membrane	1.94	0.0114	2.42	0.0405
32955	*CG14207*	*CG14207*	Unknown	unknown	unknown	2.03	0.0040	2.37	0.0033
39849	*tra*	*transformer*	Unknown	reproductive developmental process; multicellular organismal development; reproductive process in a multicellular organism; mating; sex determination; RNA metabolic process; somatic sex determination; mRNA metabolic process; sex differentiation; behavioral interaction between organisms; spliceosome assembly	spliceosome	1.54	0.0161	2.36	0.0383

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
31185	*Pgd*	*Phosphogluconate dehydrogenase (MH)^*	phosphogluconate dehydrogenase (decarboxylating) activity; NADP binding	pentose-phosphate shunt; pentose-phosphate shunt, oxidative branch	-	1.57	0.0126	2.25	0.0215
32080	*hop*	*hopscotch (MH)**	Janus kinase activity; protein-tyrosine kinase activity; non-membrane spanning protein tyrosine kinase activity; ATP binding; metallopeptidase activity; zinc ion binding	nervous system development and other anatomical structure development; sensory organ development; cell division; gamete generation; immune response; defense response; organ morphogenesis; biopolymer modification; reproductive developmental process; embryonic pattern specification	cytoplasm; cytoskeleton	1.68	0.0126	2.24	0.0238
32243	*CG4400*	*CG4400 (MH)**	Unknown	unknown	unknown	1.41	0.0242	2.21	0.0104
50392	*Lag1*	*Longevity assurance gene 1 (MH)**	sequence-specific DNA binding; transcription factor activity	regulation of transcription, DNA-dependent	integral to membrane; nucleus; plasma membrane	1.51	0.0080	2.18	0.0219
41894	*CG18522*	*CG18522*	electron carrier activity iron ion binding metal ion binding oxidoreductase activity	defense response; electron transport; nucleobase, nucleoside, nucleotide- and nucleic acid metabolic process; oxygen and reactive oxygen species- metabolic process; purine base metabolic process	-	1.70	0.0148	2.16	0.0215
47173	*Men*	*Malic enzyme (MH)**	NAD binding; malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity	malate metabolic process, tricarboxylic acid cycle	-	1.95	0.0122	2.15	0.0230
42649	*T-cp1*	*Tcp1-like (MH)**	ATP binding; ATPase activity, coupled; hydrogen-exporting ATPase activity, phosphorylative mechanism; unfolded protein binding	phagocytosis, engulfment; protein folding	chaperonin-containing T-complex; mitochondrial proton-transporting ATP synthase complex	1.48	0.0257	2.12	0.0431
33281	*S*	*Star*	Unknown	anatomical structure development; transmembrane receptor protein tyrosine kinase signaling pathway; organ development; enzyme linked receptor protein signalling pathway; organ morphogenesis; embryonic development; gamete generation; cellular macromolecule metabolic process; regulation of epidermal growth factor receptor signaling pathway; protein targeting to Golgi	Golgi apparatus, endoplasmic reticulum, integral to membrane, plasma membrane	1.60	0.0161	2.10	0.0382
39842	*mbf1*	*multiprotein bridging factor 1 (MH)**	transcription coactivator activity; methyl-CpG binding	central nervous system development, regulation of transcription from RNA polymerase II promoter, dendrite morphogenesis, open tracheal system development	cytoplasm, nucleus	1.83	0.0068	2.07	0.0052
31597	*CG3226*	*CG3226 (MH)**	Unknown	unknown	unknown	2.01	0.0180	2.06	0.0307
35246	*ref(2)P*	*refractory to sigma P (MH)**	transcription regulator activity, cysteine-type endopeptidase activity, zinc ion binding	proteolysis, viral infectious cycle	nulceus	1.83	0.0109	2.06	0.0052

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
45815	*Spn27A*	*Serpin-27A*	enzyme inhibitor activity, serine-type endopeptidase inhibitor activity	Toll signaling pathway, melanization defense response, response to symbiont, response to wounding, negative regulation of melanization defense response	-	1.74	0.0068	2.04	0.0052
42066	*cher*	*cheerio (MH)**	actin binding, structural constituent of cytoskeleton	cytoskeleton organization and biogenesis, determination of adult life span, learning and/or memory, germarium-derived female germ-line cyst encapsulation, ovarian ring canal formation, protein localization, muscle contraction	germline ring canal inner and outer rims	1.75	0.0094	2.04	0.0052
33231	*CG2789*	*CG2789 (MH)**	Benzodiazepine receptor activity, transporter activity	coenzyme metabolic process, lipid metabolic process, lipid transport, prosthetic group metabolic process	Integral to membrane, mitochondrial envelope	1.74	0.0064	2.02	0.0082
42874	*CHORD*	*CHORD (MH)**	receptor binding, zinc ion binding	signal transduction, induction of apoptosis, gamete generation	-	1.55	0.0311	2.01	0.0364
32981	*Cdc42*	*Cdc42 (MH)**	GTP binding, GTPase activity	G-protein coupled receptor protein-signaling pathway, JNK cascade, actin cytoskeleton organization and- biogenesis, axonogenesis	Intracellular, rhabdomere	1.52	0.0323	2.00	0.0471
31798	*CG12065*	*CG12065*	Unknown	unknown	unknown	1.37	0.0244	1.99	0.0094
33518	*CG17259*	*CG17259 (MH)**	ATP binding, mRNA binding, serine-tRNA ligase activity	seryl-tRNA aminoacylation	unknown	1.44	0.0287	1.99	0.0139
41054	*CG8351*	*CG8351 (MH)**	protein kinase activity, ATP binding, ATPase activity coupled, unfolded protein binding	protein amino acid phosphorylation, protein folding	chaperonin-containing T-complex	1.56	0.0210	1.96	0.0230
38145	*scf*	*supercoiling factor (MH)**	DNA topoisomerase activity, calcium ion binding, calmodulin binding	calcium-mediated signalling, dosage compensation, by hyperactivation of X chromosome, establishment and/or maintenance of chromatin architecture	polytene chromosome	1.54	0.0070	1.93	0.0094
31215	*CG2918*	*CG2918 (MH)**	ATP binding	defense response, protein folding, response to stress	-	1.52	0.0421	1.92	0.0450
41258	*CG11872*	*CG11872*	Unknown	unknown	unknown	1.29	0.0422	1.92	0.0282
32701	*CG5010*	*CG5010 (MH)**	Unknown	unknown	unknown	1.70	0.0052	1.92	0.0094
40795	*CG10267*	*CG10267*	nucleic acid binding transcription regulator activity zinc ion binding	nucleobase, nucleoside, nucleotide- and nucleic acid metabolic process regulation of transcription from RNA- polymerase II promoter transcription from RNA polymerase II- promoter	nucleus	1.41	0.0122	1.86	0.0067
326234	*l(1)G0320*	*lethal (1) G0320*	signal sequence binding, calcium ion binding	protein targeting, translation, vesicle-mediated transport	signal recognition particle- receptor complex	1.58	0.0216	1.85	0.0387
40689	*CG31549*	*CG31549*	oxidoreductase activity	lipid metabolic process	-	1.51	0.0360	1.84	0.0230

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
32722	*CG5445*	*CG5445 (MH)**	Unknown	unknown	unknown	1.61	0.0052	1.82	0.0030
43420	*CG1443*	*CG1443*	oxidoreductase activity	unknown	unknown	1.44	0.0216	1.82	0.0236
31174	*CG4199*	*CG4199 (MH)**	disulfide oxidoreductase activity	defense response, electron transport, oxygen and reactive oxygen species metabolic process, ferredoxin metabolic process	-	1.56	0.0168	1.82	0.0160
32687	*CG9086*	*Ubiquitin-protein ligase E3-alpha (MH)**	ubiquitin-protein ligase activity, zinc ion binding, protein binding	protein ubiquitination	ubiquitin ligase complex	1.45	0.0137	1.80	0.0067
37111	*GstE6*	*Glutathione S transferase E6*	glutathione transferase activity	defense response, oxygen and reactive oxygen species metabolic process, response to toxin	-	1.68	0.0420	1.79	0.0364
41503	*GstD1*	*Glutathione S transferase D1*	glutathione transferase activity	defense response, response to toxin	-	1.72	0.0052	1.79	0.0094
37110	*GstE5*	*Glutathione S transferase E5*	glutathione transferase activity	defense response, oxygen and reactive oxygen species metabolic process, response to toxin	-	1.48	0.0122	1.79	0.0094
32045	*Hsp60*	*Heat shock protein 60 (MH)**	unfolded protein binding, ATPase activity coupled, ATP binding	'de novo' protein folding, protein folding, protein refolding, protein targeting to mitochondrion, response to heat, response to stress	lipid particle, mitochondrial matrix, mitochondrion	1.55	0.0077	1.79	0.0158
31577	*CG3847*	*CG3847*	nucleic acid binding, zinc ion binding	unknown	unknown	1.56	0.0359	1.78	0.0166
40982	*CG9617*	*CG9617 (MH)**	Unknown	unknown	unknown	1.43	0.0378	1.78	0.0431
34289	*CG13117*	*CG13117*	Unknown	unknown	unknown	1.38	0.0126	1.77	0.0079
35635	*Cyp9b2*	*Cytochrome P450-9b2*	electron carrier activity, heme binding, monooxygenase activity, iron ion binding	electron transport	Membrane, microsome	1.63	0.0144	1.76	0.0236
31760	*Trxr-1*	*Thioredoxin reductase-1 (MH)**	FAD binding, antioxidant activity, oxidoreductase activity acting on NADH or NADPH disulfide as acceptor, glutathione-disulfide reductase activity, thioredoxin-disulfide reductase activity	determination of adult life span, sulfur metabolic process, thioredoxin pathway	Cytoplasm, mitochondrion	1.53	0.0142	1.75	0.0086
32458	*Top1*	*Topoisomerase 1 (MH)**	DNA topoisomerase (ATP-hydrolyzing) activity, DNA topoisomerase type I activity, nucleic acid binding	DNA replication, DNA topological change, transcription from RNA polymerase II promoter, DNA unwinding during replication, chromosome segregation and condensation, embryonic development, oogenesis, mRNA transcription	nucleus, cytoplasm, chromosome	1.50	0.0106	1.71	0.0285
43016	*CG11844*	*CG11844*	Unknown	unknown	unknown	1.50	0.0216	1.71	0.0291
33263	*drongo*	*drongo (MH)**	transporter activity	regulation of GTPase activity, transport, nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	-	1.58	0.0156	1.70	0.0067
44226	*Xbp1*	*X box binding protein-1 (H)**	transcription factor activity, sequence-specific DNA binding, protein homodimerization activity	regulation of transcription, regulation of transcription, DNA-dependent,	nucleus	1.48	0.0180	1.70	0.0082

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
42972	*CG3744*	*CG3744 (MH)*[*]	X-Pro dipeptidyl-peptidase activity, dipeptidyl-peptidase IV activity, serine-type peptidase activity	cell surface receptor linked signal transduction, proteolysis,	membrane	1.34	0.0481	1.69	0.0389
39476	*CG11267*	*CG11267 (MH)**	ATP binding ATPase activity, coupled unfolded protein binding	'de novo' protein folding	mitochondrial matrix, lipid particle, mitochondrion	1.49	0.0313	1.68	0.0307
36468	*Mp20*	*Muscle protein 20*	actin binding, calcium ion binding, structural constituent of cytoskeleton	muscle development, muscle contraction, regulation of cell shape, cell adhesion	contractile fiber	1.47	0.0189	1.68	0.0106
53578	*Jafrac1*	*Thioredoxin peroxidase 1 (H)**	antioxidant activity, glutathione peroxidase activity, thioredoxin peroxidise activity	cell redox homeostasis, oxygen and reactive oxygen species metabolic process, defense response	cytosol	1.51	0.0323	1.67	0.0248
38628	*bc10*	*bc10 (MH)**	Unknown	unknown	unknown	1.28	0.0358	1.66	0.0237
35194	*Aats-asn*	*Asparaginyl-tRNA synthetase*	ATP binding, asparagine-tRNA ligase activity, aspartate-tRNA ligase activity, mRNA binding	asparaginyl-tRNA aminoacylation, aspartyl-tRNA aminoacylation	cytoplasm	1.37	0.0421	1.64	0.0094
36760	*ATPCL*	*ATP citrate lyase (MH)**	ATP citrate synthase activity, oxygen binding	acetyl-CoA biosynthetic process, citrate metabolic process, tricarboxylic acid cycle	cytoplasm	1.56	0.0320	1.64	0.0431
37165	*Mctp*	*Multiple C2 domain and transmembrane region protein*	Unknown	unknown	-	1.26	0.0366	1.63	0.0364
32300	*CG1998*	*CG1998 (MH)**	C-4 methylsterol oxidase activity	cholesterol metabolic process	-	1.28	0.0210	1.62	0.0009
32109	*rho-4*	*rhomboid-4*	receptor signaling protein activity, receptor binding, calcium ion binding, serine-type peptidase activity	nervous system development, ectoderm development	integral to membrane, plasma membrane	1.40	0.0114	1.61	0.0094
326171	*CG31917*	*CG31917*	Unknown	unknown	-	1.42	0.0216	1.60	0.0052
43565	*Axn*	*Axin*	beta-catenin binding, signal transducer activity	Wnt receptor signaling pathway, eye-antennal disc morphogenesis, negative regulation of Wnt receptor- signaling pathway, phagocytosis,/engulfment	cytoplasm	1.42	0.0137	1.58	0.0219
35779	*CG11210*	*CG11210*	Unknown	unknown	unknown	1.25	0.0358	1.58	0.0262
38232	*Cdc37*	*Cdc37 (MH)*[*]	chaperone binding, protein tyrosine kinase activator activity, unfolded protein binding	protein folding, protein kinase cascade, regulation of progression through cell- cycle, transmembrane receptor protein tyrosine kinase -signaling pathway	cytoplasm	1.44	0.0216	1.57	0.0486
251984	*Jheh1*	*Juvenile hormone epoxide hydrolase 1*	epoxide hydrolase activity, juvenile hormone epoxide hydrolase activity	defense response, juvenile hormone catabolic process, response to toxin	membrane, microsome	1.42	0.0498	1.57	0.0447
33505	*Chd1*	*Chromodomain-helicase-DNA-binding protein (MH)*[*]	ATP binding, ATP-dependent helicase activity, chromatin binding, nucleic acid binding	chromatin assembly or disassembly, regulation of transcription from RNA polymerase II-promoter	Chromatin, polytene chromosome puff, polytene chromosome-interband, nucleus	1.31	0.0367	1.54	0.0389

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
32518	*l(1)G0022*	*lethal (1) G0022*	ATP binding, ATPase activity coupled, unfolded protein binding	protein folding	chaperonin-containing T-complex,l ipid particle	1.44	0.0085	1.53	0.0176
39167	*CG14164*	*CG14164*	Unknown	unknown	unknown	1.45	0.0070	1.51	0.0156
32128	*CG9360*	*CG9360 (MH)**	oxidoreductase activity acting on CH-OH group of donors	metabolic process	-	1.32	0.0313	1.50	0.0111
41840	*Hsc70-4*	*Heat shock protein cognate 4 (MH)**	ATP binding, ATPase activity, unfolded protein binding	RNA interference, axon guidance, nervous system development, neurotransmitter secretion, protein complex assembly, protein folding, synaptic vesicle transport, response to heat	Mitochondrion lipid particle nucleus	1.39	0.0440	1.49	0.0444
261629	*CG31352*	*CG31352 (MH)**	actin binding, structural constituent of cytoskeleton, zinc ion binding	cell motility, cytoskeleton organization and biogenesis, nervous system development ectoderm development, multicellular organismal development	-	1.34	0.0422	1.47	0.0214
37445	*Acox57D-p*	*Acyl-coenzyme a oxidase at 57D proximal*	FAD binding, acyl-CoA dehydrogenase activity, acyl-CoA oxidase activity, palmitoyl-CoA oxidase activity	electron transport, fatty acid beta-oxidation	peroxisome	1.27	0.0496	1.45	0.0431
36583	*Hsc70-5*	*Heat shock protein cognate 5 (MH)**	ATP binding, ATPase activity, unfolded protein binding	defense response, protein folding, protein targeting to mitochondrion, response to heat	mitochondrion	1.37	0.0217	1.44	0.0189
40967	*CG9636*	*CG9636 (MH)**	Unknown	unknown	unknown	1.42	0.0291	1.42	0.0100
32042	*CG2061*	*CG2061 (MH)**	G-protein coupled receptor activity	G-protein coupled receptor protein signalling-pathway	integral to membrane	1.33	0.0272	1.42	0.0119
32429	*hiw*	*highwire (MH)**	protein binding, ubiquitin-protein ligase activity, zinc ion binding	BMP signaling pathway, locomotion, negative regulation of BMP signaling pathway, negative regulation of synaptic growth at- neuromuscular junction, regulation of synaptic growth at neuromuscular-junction, protein ubiquitination	plasma membrane	1.24	0.0462	1.38	0.0285
32273	*sno*	*strawberry notch (MH)**	ATP binding, helicase activity,nucleic acid binding	Notch signaling pathway, compound eye cone cell fate commitment, embryonic development, epidermal growth factor receptor signaling pathway, imaginal disc morphogenesis, positive regulation of transcription from RNA-polymerase II promoter	nucleus	1.34	0.0359	1.31	0.0268

Table 3.2: Comparison between RMA and GCRMA 101 gene overlaps. It shows genes with expression levels (upregulated genes) having adjusted *p* value of < 0.05 (Benjamini and Hochberg's FDR <0.05) across the two algorithms and their *Drosophila* gene ontologies. * indicates genes with mouse and / human orthologues. Genes were filtered in decreasing order of their GCRMA fold changes.

Downregulated Gene Expression in *Drosophila* Head after Acute Ethanol Exposure

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P*.Value**	**FC**	**Adjusted *P*.Value**
43072	*CG11909*	*CG11909 (HM)**	alpha-glucosidase activity	monosaccharide metabolic process; polysaccharide metabolic process	alpha-glucosidase II complex	3.00	0.0047	9.29	0.0082
34452	*CG18302*	*CG18302*	lipase activity	lipid metabolic process	-	2.66	0.0080	4.69	0.0467
37170	*CG15096*	*CG15096*	high affinity inorganic phosphate:sodium symporter activity	carbohydrate metabolic process; carbohydrate transport; cation transport; extracellular transport; phosphate metabolic process; phosphate transport	integral to membrane	3.11	0.0100	4.30	0.0237
42351	*CG6300*	*CG6300*	long-chain fatty acid transporter activity; ligase activity; actin binding	metabolic process	-	2.08	0.0180	4.24	0.0296
34048	*CG13794*	*CG13794*	Unknown	Unknown	Unknown	1.85	0.0052	2.41	0.0094
42106	*CG5840*	*CG5840 (HM)**	pyrroline-5-carboxylate reductase activity	amino acid biosynthetic process; electron transport; proline biosynthetic process	-	1.62	0.0208	2.41	0.0452
39954	*Oatp74D*	*Organic anion transporting polypeptide 74D*	organic anion transmembrane transporter activity; sodium-independent organic anion transmembrane transporter activity	organic anion transport; anion transport	membrane	1.83	0.0181	2.28	0.0307
36109	*CPTI*	*mitochondrial carnitine palmitoyltransferase I (HM)**	carnitine O-palmitoyltransferase activity; acetyltransferase activity; ATP binding	amino acid metabolic process	mitochondrion	1.82	0.0240	2.25	0.0310
39391	*CG6910*	*CG6910 (HM)**	oxidoreductase activity	carbohydrate metabolic process; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	-	1.91	0.0356	2.21	0.0446
35913	*ana*	*anachronism*	Unknown	negative regulation of neuroblast proliferation	extracellular region	1.59	0.0085	2.17	0.0094
37573	*Px*	*plexus*	Unknown	imaginal disc-derived wing vein morphogenesis	nucleoplasm	1.69	0.0040	2.07	0.0201
34436	*CG5322*	*CG5322*	alpha-mannosidase activity; hydrolase activity, hydrolyzing N-glycosyl compound	carbohydrate metabolic process; mannose metabolic process	lysosome	1.49	0.0390	2.04	0.0364
34370	*Rsf1*	*Repressor splicing factor 1*	mRNA binding; nucleotide binding	negative regulation of nuclear mRNA splicing via spliceosome	nucleus	1.67	0.0105	2.02	0.0079
34730	*CG16820*	*CG16820*	Unknown	Unknown	Unknown	1.34	0.0381	2.00	0.0219
34152	*CG17292*	*CG17292*	triacylglycerol lipase activity	lipid metabolic process	-	1.48	0.0161	1.99	0.0094
38325	*CG16986*	*CG16986*	Unknown	Unknown	Unknown	1.63	0.0320	1.99	0.0450
39020	*GNBP3*	*Gram-negative bacteria binding protein 3*	Pattern recognition receptor activity; Gram-negative bacterial binding; glucosidase activity	response to fungus; defense response; polysaccharide metabolic process	-	1.55	0.0294	1.95	0.0139
39031	*CG5288*	*CG5288 (HM)**	galactokinase activity; ATP binding	monosaccharide metabolic process; carbohydrate phosphorylation; galactose metabolic process; phosphorylation	cytoplasm	1.71	0.0070	1.89	0.0219
38772	*CG14823*	*CG14823*	Unknown	Unknown	Unknown	1.49	0.0390	1.89	0.0411

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P*.Value**	**FC**	**Adjusted *P*.Value**
41786	*Smp-30*	*senescence marker protein-30 (HM)**	calcium ion binding	anterior/posterior axis specification; calcium-mediated signaling; multicellular organismal development; intracellular signaling cascade; signal transduction	-	1.38	0.0301	1.86	0.0376
38871	*Hn*	*Henna (HM)**	phenylalanine 4-monooxygenase activity; tryptophan 5-monooxygenase activity; iron ion binding	L-phenylalanine catabolic process; eye pigment biosynthetic process; signal transduction; phagocytosis, engulfment	-	1.61	0.0100	1.86	0.0209
3771965	*CG9510*	*CG9510*	argininosuccinate lyase activity	Unknown	-	1.54	0.0287	1.86	0.0285
37480	*HmgZ*	*Hmg protein Z*	DNA binding; chromatin binding; transcription regulator activity	chromatin assembly or disassembly; regulation of transcription from RNA polymerase II promoter	nucleus	1.58	0.0070	1.81	0.0067
40122	*CG9295*	*Cuticular protein 76Bc*	structural constituent of chitin-based cuticle	Unknown	-	1.45	0.0422	1.81	0.0383
42294	*Cyp12a4*	*Cyp12a4*	electron carrier activity; oxidoreductase activity; heme binding; iron ion binding; monooxygenase activity	response to insecticide; electron transport	mitochondrion	1.50	0.0161	1.81	0.0086
41273	*Hth*	*homothorax (HM)**	transcription factor activity; sequence-specific DNA binding; DNA binding	anatomical structure development; organ morphogenesis; organ development; segment specification; sensory organ development; head segmentation; regulation of metabolic process; leg disc proximal/distal pattern formation; transcription from RNA polymerase II promoter; leg morphogenesis; specification of segmental identity, head; central nervous system development; specification of segmental identity, antennal segment; compound eye development	Nucleus	1.53	0.0300	1.80	0.0258
42234	*CG7695*	*CG7695*	Unknown	Unknown	Unknown	1.37	0.0313	1.76	0.0082
37294	*CG10444*	*CG10444*	Sodium-dependent multivitamin transmembrane transporter activity; cation transmembrane transporter activity	cation transport; coenzyme metabolic process; extracellular transport; prosthetic group metabolic process	membrane	1.45	0.0080	1.76	0.0080
34897	*CG15261*	*UK114*	Unknown	protein folding; negative regulation of protein biosynthetic process; regulation of translation	-	1.43	0.0161	1.75	0.0219
41911	*CG4699*	*CG4699*	Unknown	Unknown	-	1.45	0.0375	1.73	0.0079
35837	*Cyp6a13*	*Cyp6a13*	electron carrier activity; oxidoreductase activity; heme binding; iron ion binding; monooxygenase activity	steroid metabolic process; electron transport	Membrane; microsome	1.45	0.0298	1.73	0.0181
35419	*Ac3*	*Ac3 (HM)**	adenylate cyclase activity	cAMP biosynthetic process; G-protein coupled receptor protein signaling pathway; cyclic nucleotide metabolic process; intracellular signaling cascade	integral to membrane	1.48	0.0070	1.73	0.0052

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
38864	*CG12262*	*CG12262 (HM)**	acyl-CoA dehydrogenase activity	fatty acid beta-oxidation; acyl-CoA metabolic process; electron transport	lipid particle;mitochondrion	1.44	0.0161	1.72	0.0346
35190	*Ddc*	*Dopa decarboxylase (HM)**	aromatic-L-amino-acid decarboxylase activity	dopamine biosynthetic process from tyrosine; serotonin biosynthetic process from tryptophan; learning and/or memory; catecholamine metabolic process; courtship behavior; eclosion rhythm; cuticle development; melanin biosynthetic process; pigmentation during development; growth	-	1.50	0.0097	1.72	0.0067
42762	*CG4408*	*CG4408*	metallocarboxypeptidase activity; carboxypeptidase A activity	proteolysis	-	1.35	0.0393	1.69	0.0349
34313	*yip2*	*yippee interacting protein 2*	Acetyl-CoA C-acyltransferase activity	fatty acid beta-oxidation	-	1.39	0.0216	1.69	0.0215
32037	*CG1537*	*CG1537*	Unknown	Unknown	Unknown	1.30	0.0381	1.69	0.0086
40901	*alpha-Est7*	*α-Esterase-7*	carboxylesterase activity	Unknown	-	1.48	0.0161	1.69	0.0106
33196	*Smo*	*Smoothened (HM)**	G-protein coupled receptor activity; protein binding; transmembrane receptor activity; hedgehog receptor activity; non-G-protein coupled 7TM receptor activity	anatomical structure development; cell communication; organ development; signal transduction; organ morphogenesis; system development; sensory organ development; regionalization; cell surface receptor linked signal transduction; embryonic pattern specification; Wnt receptor signaling pathway; G-protein coupled receptor protein signaling pathway; cell cycle	Endosome; integral to membrane; plasma membrane	1.35	0.0216	1.68	0.0108
41272	*Cyp12e1*	*Cyp12e1*	electron carrier activity; heme binding; iron ion binding; monooxygenase activity; structural constituent of ribosome	electron transport; translation	Mitochondrion; ribosome	1.32	0.0330	1.68	0.0262
36366	*CG8550*	*CG8550*	metalloendopeptidase activity; neprilysin activity; zinc ion binding	proteolysis	membrane	1.30	0.0161	1.65	0.0130
41686	*CG9312*	*CG9312*	Unknown	Unknown	Unknown	1.35	0.0183	1.65	0.0030
33541	*CG9663*	*CG9663*	ATPase activity, coupled to transmembrane movement of substances; transporter activity; ATP binding	lipid metabolic process; lipid transport	ATP-binding cassette (ABC) transporter complex	1.40	0.0216	1.62	0.0452
31184	*CG3835*	*CG3835 (HM)**	oxidoreductase activity	carbohydrate metabolic process; electron transport	-	1.36	0.0297	1.59	0.0157

Genes			*Drosophila* Gene Ontology			RMA		GCRMA	
Entrenz ID	**Symbol**	**Name**	**Function**	**Process**	**Component**	**FC**	**Adjusted *P.*Value**	**FC**	**Adjusted *P.*Value**
7481	*HmgD*	*High mobility group protein D*	AT DNA binding; DNA bending activity	establishment and/or maintenance of chromatin architecture; regulation of transcription, DNA-dependent; dendrite morphogenesis; muscle development	Colocalizes with nuclear chromatin	1.37	0.0161	1.55	0.0154
40513	*CG12768*	*CG12768*	Unknown	Unknown	Unknown	1.32	0.0209	1.51	0.0339
34044	*TepII*	*Thiolester containing protein II (HM)**	wide-spectrum protease inhibitor activity; serine-type endopeptidase inhibitor activity	antibacterial humoral response; phagocytosis, engulfment; defense response to Gram-negative bacterium	-	1.36	0.0165	1.49	0.0258
32695	*CG4991*	*CG4991*	amine transmembrane transporter activity	amino acid transport	membrane	1.30	0.0494	1.48	0.0450
34315	*CG4598*	*CG4598 (HM)**	dodecenoyl-CoA delta-isomerase activity; hydro-lyase activity	fatty acid beta-oxidation	-	1.33	0.0483	1.47	0.0449
41767	*Cys*	*Cystatin-like*	cysteine protease inhibitor activity	Unknown	-	1.39	0.0489	1.46	0.0398
42364	*Arc42*	*Arc42 (HM)**	RNA polymerase II transcription mediator activity; acyl-CoA dehydrogenase activity	Transcription initiation from RNA polymerase II promoter; acyl-coa metabolic process; electron transport; dendrite morphogenesis	mediator complex	1.24	0.0496	1.42	0.0431
41067	*CG11963*	*CG11963 (HM)**	succinate-CoA ligase (ADP-forming) activity; ATP binding	tricarboxylic acid cycle	succinate-CoA ligase complex (ADP-forming	1.27	0.0216	1.35	0.0162
31721	*CG2233*	*CG2233*	Unknown	Unknown	Unknown	1.28	0.0216	1.29	0.0094
34704	*CG9928*	*CG9928*	Unknown	Unknown	Unknown	1.27	0.0262	1.28	0.0391

Table 3.3: Comparison between RMA and GCRMA 54 gene overlaps. It shows genes with expression levels (downregulated genes) having adjusted *p* value of < 0.05 (Benjamini and Hochberg's FDR < 0.05) across the two algorithms and their *Drosophila* gene ontologies. * indicates genes with mouse and / human orthologues. Genes were filtered in decreasing order of their GCRMA fold changes.

(b) Non stringent analysis-P 0.1

In the non-stringent analysis, 313 genes met the criteria for differential expression (211 upregulated and 102 downregulated). This P-0.1 list of genes was used in the following further analyses:

Functional Clusters: 211 gene symbols were mapped to 202 DAVID IDs using the DAVID software (Dennis et al., 2003). Twenty four functional clusters containing at least one significant annotation term ($P \leq 0.1$) were identified and these were further reduced to eight by eliminating genes clustered mainly according to cellular component and by removing clusters with less than three annotation terms which otherwise might results in overestimation of the true functional size (Table 3.4 and Appendix B.1). For the downregulated genes, 102 of the Gene symbols mapped to 98 DAVID IDs (the difference is probably due to a slight difference in some *Drosophila* gene symbols between DAVID and flybase). Fourteen functional clusters containing at least one significant annotation term ($P \leq 0.1$) were identified and these were further reduced to ten by eliminating genes clustered mainly according to cellular component and by removing clusters with less than three annotation terms which otherwise might result in overestimation of the true functional size (Table 3.4 and Appendix B.1 and B.2).

Table 3.4: The first three significance (*p* value) DAVID functional clusters for up-and down-regulated genes. The complete functional clusters can be seen in Appendix B.1 and B.2. Annotation terms reaching significant enrichment scores (EASE score $\leq$ 0.1) are reported. N: number of genes in functional theme, n: number of genes with enriched annotation term for all differentially expressed genes clustered by DAVID

Functional clusters of ethanol-affected genes in *Drosophila* head

Category	Functional theme	GO annotation term	N	n	*P*
Upregulated					
GOTERM_BP_ALL		protein folding		23	5.09E-16
GOTERM_BP_ALL		response to biotic stimulus		31	7.77E-11
GOTERM_BP_ALL		defense response		30	2.20E-10
GOTERM_BP_ALL	Response to stimulus	response to heat	51	10	1.45E-08
GOTERM_BP_ALL		response to temperature stimulus		10	6.36E-08
GOTERM_BP_ALL		response to abiotic stimulus		23	7.74E-07
GOTERM_BP_ALL		response to stress		20	1.94E-06
GOTERM_BP_ALL		response to stimulus		41	4.22E-06
GOTERM_BP_ALL		protein folding		23	5.09E-16
GOTERM_MF_ALL		unfolded protein binding		17	2.07E-14
GOTERM_CC_ALL		chaperonin-containing T-complex		7	1.26E-10
GOTERM_MF_ALL		adenyl nucleotide binding		33	6.31E-08
GOTERM_MF_ALL		purine nucleotide binding		36	2.75E-07
GOTERM_MF_ALL		ATP binding		31	3.01E-07
GOTERM_MF_ALL	Protein folding	nucleotide binding	65	39	3.74E-07
GOTERM_MF_ALL		ATPase activity		17	1.23E-04
GOTERM_MF_ALL		pyrophosphatase activity		20	2.55E-04
GOTERM_MF_ALL		ATPase activity, coupled		15	3.13E-04
GOTERM_MF_ALL		hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides		20	3.58E-04
GOTERM_MF_ALL		hydrolase activity, acting on acid anhydrides		20	3.65E-04
GOTERM_CC_ALL		Cytosol		15	3.95E-04
GOTERM_MF_ALL		nucleoside-triphosphatase activity		19	5.78E-04
GOTERM_MF_ALL		glutathione transferase activity		9	3.30E-08
GOTERM_MF_ALL		transferase activity, transferring alkyl or aryl (other than methyl) groups		9	1.59E-06
GOTERM_BP_ALL	Defense response	response to toxin	32	11	1.60E-05
GOTERM_BP_ALL		response to chemical stimulus		15	3.61E-04
GOTERM_BP_ALL		oxygen and reactive oxygen species metabolism		7	9.39E-04
Downregulated					
GOTERM_MF_ALL		oxidoreductase activity		17	3.28E-05
GOTERM_BP_ALL		electron transport		12	1.92E-04
GOTERM_BP_ALL		generation of precursor metabolites and energy		13	1.26E-03
GOTERM_MF_ALL	Oxidoreductase activity	transporter activity	36	18	8.05E-03
GOTERM_BP_ALL		Transport		25	1.26E-02
GOTERM_BP_ALL		Localization		27	2.25E-02
GOTERM_BP_ALL		establishment of localization		26	2.91E-02
GOTERM_BP_ALL		lipid metabolism		12	1.52E-03
GOTERM_BP_ALL	Lipid metabolism	cellular lipid metabolism	12	9	4.37E-03
GOTERM_BP_ALL		fatty acid metabolism		5	6.70E-03
GOTERM_MF_ALL		acyl-CoA dehydrogenase activity		3	4.05E-03
GOTERM_BP_ALL		fatty acid metabolism		5	6.70E-03
GOTERM_BP_ALL	Catalytic activity	acyl-CoA metabolism	8	3	7.56E-03
GOTERM_MF_ALL		oxidoreductase activity, acting on the CH-CH group of donors		3	1.78E-02

The gene annotations from the P-0.05 and P-0.1 analyses have successfully shown that heat shock proteins and other stress related genes showed increased response to acute ethanol exposure. This is consistent with the previous reports that heat shock proteins are induced by exposure to ethanol in other model systems (Kwon et al., 2004; Nishida et al., 2000; Alexandre et al., 2001). Moreover, genes involved in reproduction were upregulated in this study, indicating that *Drosophila* may direct its available resources toward reproduction, resulting in a transcriptional increase of these genes. Further, the upregulation of genes involved in multiple signaling cascades may have led to the altered gene expression of the *Drosophila* head exposed to acute ethanol (Spanagel, 2009). Other genes involved in metabolism were differentially expressed and this has been suggested to indicate a form of compensatory defense response aiming at rapidly ensuring sufficient ATP production defence from reactive oxygen species (ROS) (Spanagel, 2009). Finally, genes with alterations in transport activities might be a consequence of the oxidative stress caused by ethanol.

Pathways and networks: The DAVID analysis described above also allowed mapping the genes of interests to the known regulatory and metabolic pathways from the KEGG database, the results of which are shown in Figure 3.15 b. In parallel, KEGG spider (Antonov et al., 2008) was also used to query the KEGG database and infers the metabolic pathways and network that are enriched from the P-0.1 list of genes. The 211 P-0.1 upregulated gene symbols mapped to 30 KEGG metabolic reaction networks and three enriched KEGG pathways were identified. For the P-0.1 downregulated genes, 102 of the gene symbols mapped to 37 KEGG metabolic reaction network and nine enriched KEGG pathways were identified. The pathways discovered by KEGG spider are shown in Figure 3.15a and are comparable to those obtained in the DAVID analysis (Figure 3.15 b). For the metabolic network analysis, the whole P-0.1 differentially expressed genes were mapped to 67 KEGG metabolic reaction pathways using the KEGG spider software and three enriched sub-network models were identified (Figure 3.16 and data not shown).

As shown in Figure 3.15 both DAVID and KEGG spider showed that metabolic signaling drives ethanol-induced gene expression in *Drosophila* head, as genes acting in the glutathione metabolism, metabolism of xenobiotics by cytochrome P450, fatty acid metabolism, propanoate metabolism, limonene and pinene degradation and gamma hexachlorocyclohexane degradation are overrepresented at a *p value* ≤0.05 (Figure 3.15). In addition, KEGG spider highlights four other significant pathways (*p value* < 0.13) involving naphthalene and anthracene degradation, galactose metabolism, valine, leucine and isoleucine degradation and tryptophan metabolism. For the gene network model, the most enriched sub-network model (*p* value < 0.02) shown in Figure 3.16 covered 33 genes mapped to the KEGG metabolic reaction pathway. This was followed by the two other models covering 16 (*p* value < 0.05) and 56 (*p* value < 0.06) genes that were mapped to the KEGG metabolic reaction pathway (data not shown).

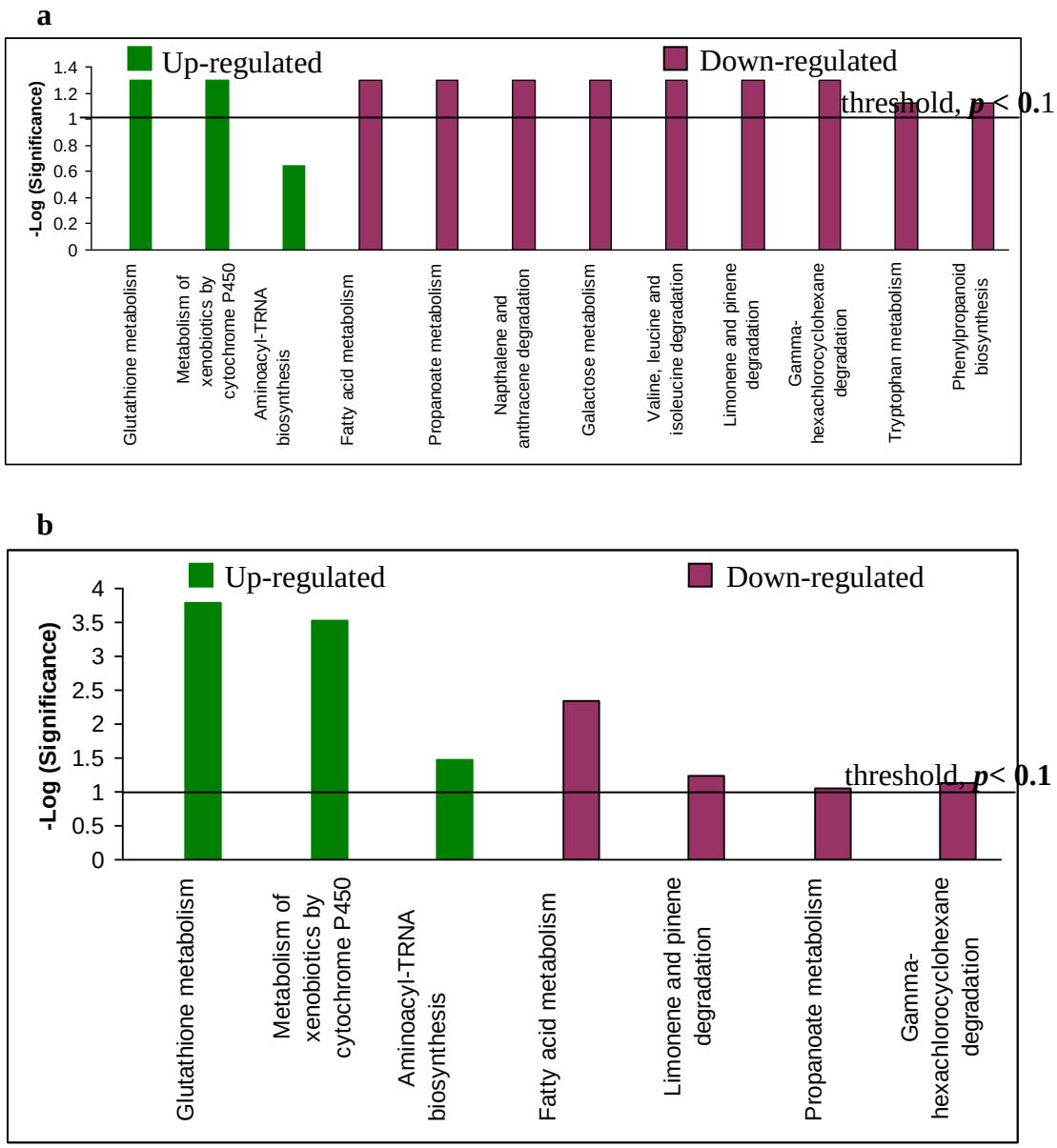


Figure 3.15: (**a)** Global pathway analysis: comparison of up- and down-regulated gene datasets. Each dataset was analysed by the KEGG spider analysis software (Antonov et al., 2008) In total 12 metabolic pathways regulated by alcohol were discovered based on their significance. The significance is expressed as a *P* value that is calculated using a robust Monte Carlo Simulation statistical procedure (Antonov et al., 2008). (**b)** Global pathway analysis: comparison of up- and down-regulated gene datasets. Each dataset was analysed by the NIH DAVID analysis software (Dennis et al., 2003). In total 7 metabolic pathways regulated by alcohol were discovered based on their significance. The significance is expressed as a *P* value that is calculated using a Fisher's exact test. The horizontal line depicts a significance threshold on the graphs.

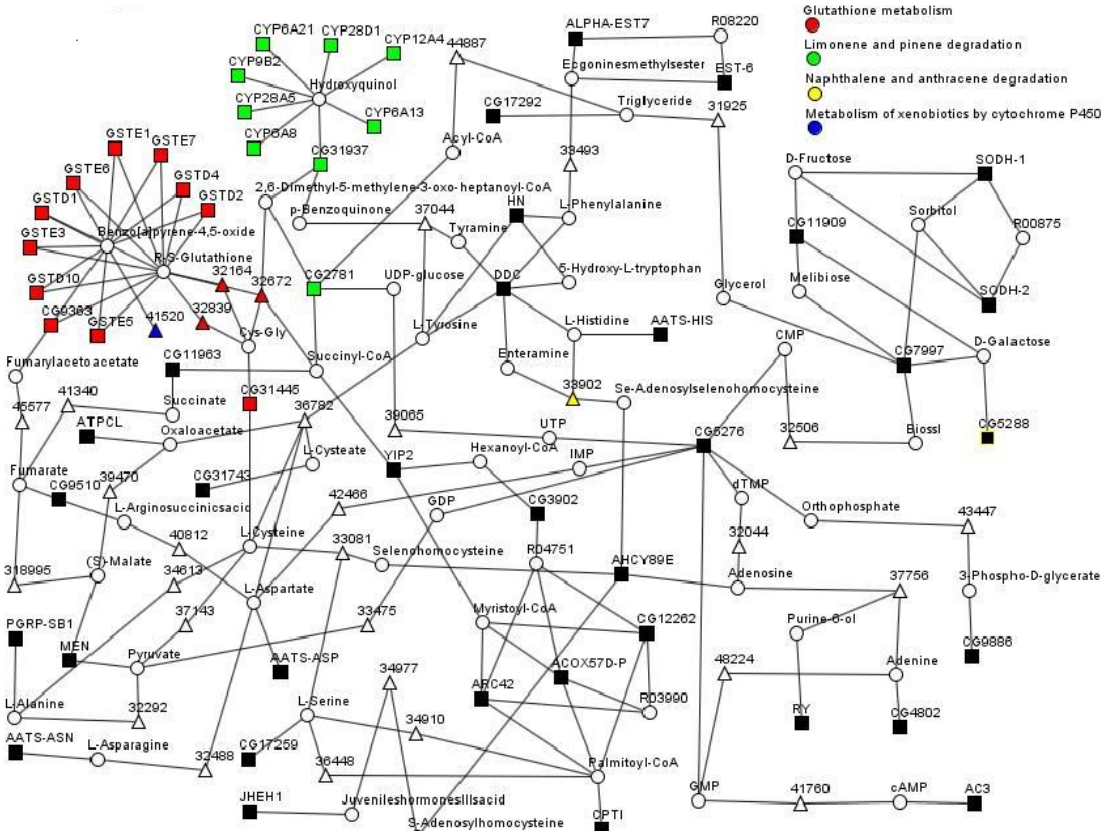


Figure 3.16: A network model of common up- or down-regulated genes in *Drosophila* head exposed to acute ethanol. The dataset was analysed by the KEGG spider analysis software (Antonov et al., 2008). Sixty-seven genes were mapped to KEGG metabolic reaction pathways; the model shown is the most significant network and covers 53 genes (p-value < 0.02). Genes from the input list are indicated by rectangles, intermediate genes by triangles and chemical compound by circles. Different colours specify different KEGG canonical pathways. Black indicates genes not directly involved in any of the pathways but forming part of the network.

3.3.4 Differentially Expressed Genes and Pathways Discussion

The data from both P-0.05 and P-0.1 analysis suggest that *Drosophila* head responds to ethanol stress by upregulation of genes involved in general stress pathways and several signaling cascades including protein folding, detoxification, immune response and inter and intra-cellular signaling and downregulation of genes encoding many metabolic pathways including fatty acid and lipid metabolism. Several genes involved in transcriptional regulation and proteolysis were also regulated. Such regulation support the view that a single exposure to ethanol has the potential to evoke significant changes in the protein composition of the cell through altered transcriptional regulation and proteolysis targeted at rapidly adapting cellular metabolism to the effects of ethanol intoxication (Morozova et al., 2006). Thus the computational analysis has shown that these data sets are useful to determine gene sets (families) and pathways involved in alcohol response.

The results obtained in the current study are validated by the identification of pathways or gene sets known to be critical for ethanol related response in *Drosophila* such as signal transduction, lipid metabolism, immune and stress pathways. In addition, a comparison of genes obtained from the P-0.05 list with that obtained by Morozova et al., 2006 and 2007 revealed that 64 (i.e. 41.3%) of these genes were previously reported in both studies of gene expression in *Drosophila* (Appendix B.3). However, more importantly, this study has found genes that have never been associated with ethanol response in *Drosophila* before. Most prominent are the genes encoding the small heat shock protein *Hsp26*, *highwire* (*hiw*), and a member of the endothelial differentiation-related factor families, *multiprotein bridging factor* (*mbf1*). Small heat shock proteins serve as molecular chaperones to protect proteins from various insults (stressors) (Joanisse et al., 1998). *Hiw* functions in protein ubiquitination by regulating synaptic growth and development (DiAntonio et al, 2001, Wan et al., 2005). *mbf1* is a transcription factor involved in oxidative stress response (Jindra et al. 2004).

In addition, such detailed analyses of the effect of acute ethanol regulation in *Drosophila* head has revealed that ethanol can activate genes encoding pathways contributing to many functions and processes, a suggestion consistent with many

other experimental findings. For example, the activation of such genes encoding (but not limited) to stress response, detoxification, transcription regulation, proteolysis, ethanol metabolism, neurotransmitter secretion, synaptic transmission, immune response, and inter and intra- cellular signaling, have been found in a number of microarray studies in both flies and mammalian models (Morozova et al., 2006 and 2007, Urizar et al., 2007, Worst and Vrana, 2005). Strikingly, a considerable number of genes with similar and distinct functions appear to be regulated by ethanol and it is of special interest that some of these genes are involved in axon guidance (*Cdc 42, Cys*) and changes in neuronal architecture (*Ddc, ana, hth, Hsp83*) and synaptic structure (*hiw,Hsp83, GstE1,Ddc, tra, Cys, DnaJ-1,Hsc70-4*).

This study has also discovered a large group of ethanol-regulated transcripts that have previously been identified as under the control of circadian clock in *Drosophi*la (Claridge-Chang, et al., 2001; Cirelli et al., 2005), suggesting some underlying shared mechanisms. Notable are genes involved in detoxification (*cytochrome P450s*), response to cellular stress (*glutathione S-transferases*, *Hsp83*), metabolism (*Men*, *ATPCL*, *Pgd*), neurogenesis (*ana*) and immune response. For instance, this study has confirmed the involvement of *Hsp83* in *Drosophila* ethanol response (see Chapter 4, section 4.5.6 and Chapter 5, section 5.4.2). Consistent with the role of this gene in circadian rhythm was the finding that flies carrying a mutation for the heat-shock protein *Hsp83* (*Hsp83*^{08445}) showed increase homeostatic response and died after sleep deprivation (Shaw et al., 2002). In addition, transcriptional response to alcohol has shown that a mutation in the cytosolic malic enzyme (*Men*), affects the flies' tolerance to ethanol (Morozova et al., 2006). Indeed, drugs of abuse (e.g. ethanol) have been reported to clearly induce specific expression changes in clock genes in the brain (Perreau-Lenz and Spanagel, 2008).

As previously noted ethanol treatments in *Drosophila* appear to result in the induction of some of the Hsp genes (Scholz et al., 2005, Morozova et al., 2006). We identified nine ethanol-regulated genes encoding heat shock proteins that mediate stress responses from our analysis (Table 3.1) and validated two of them in the sedation and recovery assay (see Chapters 4 and 5 for results). Four of these heat

shock genes including *Hsp70cb* and *Hop* (*Hsp70/Hsp90* organising protein) have both mouse and human orthologues (Table 3.1). Notably, immediate upregulation for transcripts that mediate stress responses including *l(2)efl* and *Hsp70* have been recently reported (Morozova *et al.*, 2006) to be implicated in acute ethanol response in *Drosophila*. Indeed, *Hsp70* has been reported to be induced in adult flies exposed to ethanol (Scholz et al., 2005). Similarly, this stress gene has been shown to be upregulated in various regions of the brain of male Wistar rats fed with acute as well as chronic ethanol for 12 weeks (Calabrese, *et al.*, 1996) while immunohistochemical detection revealed elevated *Hsp70* in livers of alcoholic patients (Omar, *et al.*, 1990). In addition, a member of the *Hsp70* encoding protein, *Hsc70* also identified in our microarray analysis is involved in regulating the release of neurotransmitter through the uncoating of clathrin-coated vesicles and regulation of soluble NSF attachment protein receptor (SNARE) complex-associated protein interactions alongside with cysteine-string protein (flybase, Zinsmaier and Bronk, 2001). Further, the mechanism of neuronal adaptation to ethanol has shown that the concentration of ethanol observed in heavy drinkers produced significant increases in *Hsc70* mRNA and protein suggesting the possible role of *Hsc70* in neuronal adaptation to ethanol tolerance and dependence in alcoholics (Miles et al., 1991).

It is becoming increasingly clear that ethanol plays a significant role in regulating cell signaling pathways that are central to normal and abnormal brain function. Ethanol like other additive drugs activates signal transduction pathways that regulate brain gene expression (Torres et al., 1999). A considerable body of evidence demonstrating how ethanol affects a wide range of signaling cascades is well documented in heavy drinkers (Mckillop and Schrum, 2006). The identification of multiple genes associated with multiple signaling cascades in the response to ethanol exposure in *Drosophila* is, therefore, not only of particular interest but also reflects the reliability of our methods and validity of our results. *Tetraspanin 42EK, Axin, hopscotch, star, CHORD* and *Cdc 42,* and *Axin* are all critically implicated in signaling pathways and also involved in nervous system development (Table 3.2). For instance, *star* has been implicated in the production of an activated ligand for the epidermal growth factor (EGF) receptor signaling pathway (Pickup and Banerjee, 1999), tetraspanin proteins regulate cell motility and signaling in the

brain (reviewed in Hemler, 2005) while Axin family proteins mediate a set of conserved biochemical processes that suppresses Wnt/Wingless (Wg) signaling in *Drosophila* (Willert et al., 1999). Similarly, Axin gene has been shown to negatively regulate the Wnt signaling pathway by interacting with GSK-3β protein from a rat brain and a β-catenin and mediate signal from a GSK-3β to β-catenin (Ikeda et al., 1998).

The gene expression changes in metabolism implicate many pathways including glutathione metabolism, fatty acid metabolism, gamma- hexachlorocyclohexane degradation and tryptophan metabolism as some of the significantly altered metabolic pathways in the *Drosophila* head exposed to acute ethanol. Glutathione metabolism is the most significantly altered upregulated pathway in our analyses. The alterations in the glutathione metabolic pathways are markedly apparent in Figure 3.16. All the genes encoding this pathway were clearly clustered together. *Glutathione S-transferases* (*GSTs*) are a family of multifunctional dimeric proteins involved in xenobiotic metabolism, drug transformation and protection against peroxidase damage (Tsuchida and Sato, 1992). Reports have indicated that increase in expression of these detoxification enzymes constitute a special response for development of chemical resistance in many insect species and mammalian systems (for review: Waxman and Azaroff, 1992). At least two classes of GSTs proteins-the GST D isozymes and GST-2, have been found in *Drosophila* and the activities of these GSTs are present in the adult flies at a level comparable to those of mammals (Tang and Tu, 1994). *Drosophila gstD* genes which encode a family of GST D isozymes encode a new family of GSTs with little sequence homology to mammalian GST (Tang and Tu, 1994). Interestingly, all the GST genes found in the list were up-regulated suggesting their possible role not only in *Drosophila* physiological responses to ethanol but also their involvement in ethanol metabolism. Interestingly, several glutathione genes have been recently reported to be differentially expressed in a similar microarray experiment employing *Drosophila* in alcohol research (Urizar et al., 2007). In a related microarray research, a very large number of genes involved in glutathione metabolism were identified in the brain of ethanol-preferring rats when compared to non-preferring rats (Bjork et al., 2006), indicating a possible role of GSTs in the development of alcohol preference.

Fatty acid metabolism is the most significantly downregulated pathway in *Drosophila* head exposed to acute ethanol (Figure 3.15 a and b). Genes in this pathway include *Yippee 2* (*yip2*), *mitochondrial carnitine palmitoyltransferase* (*CPTI*) and *Arc42*. The discovery of this most down-regulated pathway in both DAVID and KEGG spider analyses is very important considering the known relationship of this pathway with alcohol dependence in man. Notably, transcriptional response to alcohol exposure in *Drosophila* identified multiple enzymes associated with fatty acid biosynthesis (Morozova et al, 2006). Indeed, several lines of evidence suggest that the fatty acid compositions of lipids are important to ethanol tolerance in *Drosophila* larvae (Swanson, et al., 1995).

Gamma hexachlorocyclohexane degradation, also known as lindane, is also significantly downregulated in the list. Genes encoding this pathway in both DAVID and KEGG spider analysis are mostly cytochrome P450 enzymes encoded. A closer look at these genes in Figure 3.16 revealed that they were indeed clustered together suggesting that they are co-ordinately regulated. Many of these have been reported in a similar microarray experiment using *Drosophila* in alcohol research (Morozova et al., 2006, Urizar et al., 2007). These enzymes have a wide range of biological functions, including drug metabolism, detoxification of xenobiotic compound, electron transport, and cholesterol metabolism. Further, coordinated regulation of cholesterol and fatty acid synthesis has been reported (Gibbons, 2003). Lindane has also been shown to increase serum cholesterol and very-low-density lipoprotein (VLDL) levels upon administration (Ghazalpour et al., 2005 and references there-in). Interestingly, pathway level analysis of gene expression using singular value decomposition has shown that lindane degradation is the most strongly differentially expressed pathway in airway epithelium of human smokers compared with non-smokers (Tomfohr et al., 2005). Thus, the current study has again highlighted the possible shared pathways between nicotine and alcohol dependence.

Lastly, several genes occur in multiple times in some of the metabolic pathways discovered using both DAVID and KEGG spider (for examples *cytochrome P450s*, *glutathiones*, *yip2* and *Arc42*). At least one of these genes is involved in valine,

leucine, and isoleucine degradation, propanoate metabolism, fatty acid metabolism, naphthalene and anthracene degradation, limonene and pinene degradation, glutathione metabolism, phenylpropanoid biosynthesis, metabolism of xenobiotics by cytochrome P450 and tryptophan metabolism (ten out of the 12 pathways). Furthermore, it is of interest that some of these pathways are interconnected. Thus, it appears that one way through which alcohol exerts it metabolic effects on the brain is through the dysregulation of the tricarboxylic acid (TCA) cycle. This is because, in all of the 12 differentially regulated pathways identified, four pathways were related via the TCA cycle. For example, propanoate metabolism and valine, leucine and isoleucine degradation can be converted to propionyl CoA and then to succinyl CoA before entering the TCA. In a similar vein, Tryptophan metabolism can be converted into pyruvate metabolism before entering the TCA while free fatty acid metabolism from glycerolipid metabolism can directly enter the TCA.

3.3.5 Temporal gene expression

Gene-expression intensity data collected from the time course microarray data are aimed at shedding light on how transcriptional response is modulated over a biologically relevant time course of acute ethanol treatment. This approach is also expected to test whether relationship exists between transcriptional response and biological function.

In this analysis, 108 genes (excluding genes not available and gen duplicates) were found to be differentially expressed (at a FDR of 0.01) with respect to one or more time points. Figure 3.17a depicts the expression values and gene information of these data in a heat map. Gene expression data comparison of variances using Levene's test (an inferential statistic used to assess the equality of variance in different samples) (Carroll and Schneider, 1985) at an alpha level of 0.05 showed that there are statistically significant variations in gene expression at all the time points ($p < 0.001$). This was also confirmed by a 1-way ANOVA assuming equality of variance (p=7.48E-18). However, a comparative analysis of the gene expression data for all the 108 genes at various time points in pair-wise manner revealed the fraction with shared variance (Table 3.5). Significant correlations were found for gene expression estimates for some of the pair-wise comparisons including 0 and 0.5 h, 0.25 and 1h, 0.5 and 2 h, 1 and 3 h, 2 and 4 h and 3 and 4 h.

	0 h	0.25 h	0.5 h	1 h	2 h	3 h	4 h
0 h		0.186	**0.682**	0.093	**0.312**	**-0.202**	0.095
0.25 h			**0.512**	**0.777**	0.074	**0.256**	-0.162
0.5 h				**0.598**	**0.579**	**0.213**	**0.293**
1 h					**0.438**	**0.670**	0.030
2 h						**0.688**	**0.592**
3 h							**0.346**

Table 3.5: Pearson correlation coefficient similarity matrix on the time-dependent gene expression estimates. It shows pair-wise comparisons of the various time points gene expression values. Significant values using Bartlett's sphericity testing method (Manoukian et al., 1986) at the level of significance, p=0.05 are shown in bold.

3.3.6 Identification of time-dependent gene classes in *Drosophila* head

The 108 differentially expressed genes obtained above were further classified into groups based on their gene expression profiles using STEM cluster software (Ernst and Bar-Joseph, 2006). The STEM clustering method was used to partition the data according to gene expression patterns through time (class discovery). Through this method, classes of ethanol-responsive genes over time were identified. STEM automatically group the 108 ethanol-affected genes into 18 classes (with 8 significant classes i.e. clusters containing higher number of genes than assigned to the model profiles) according to their expression profiles (Figure 3.17b). Consequently, the prominent ethanol-induced genes are easily identified in the clustering results. The 8 significant classes are discussed below.

Class 1

Class 1 consists of genes whose transcription levels increased at very early stages and were maintained in an undulating manner before reaching a peak at 2 h. Class 1 is composed of 19 genes including those involved in enzyme activities (e.g. *kraken* and *Pgd*), transcription co-activator (e.g. *mbf1*) and unfolded protein binding (*Cct5* and *Hsp83*).

Class 2

Class 2 genes are those whose expression levels at 2 h were higher than those at other time points and the expression levels at time points 3 and 1 h were lower than those at 2 h. Class 2 is composed of 18 genes, with most of these genes involved in protein binding (e.g. *Hsp23, NAT1, Gp93, sec63, hfw* and *CG4845*).

Class 3

Class 3 genes are those showing increase in transcript abundance with a peak of activity at 4 h following acute ethanol exposure. Between 0 and 4 h the genes displayed undulating patterns of transcriptional expression. Class 3 has 16 genes including *Men*-a malate enzyme dehydrogenase involved in metal ion and NAD binding, *Obp99d*-an odorant binding protein and *Jheh1* involved in juvenile hormone epoxide hydrolase activity. Nearly all the genes in this class encode metabolic and detoxifying enzymes.

Class 4

Class 4 genes are those showing a gradual increase in the expression levels reaching peak between 2 and 3 h and then fell abruptly to the control levels at 4 h. Class 4 is composed of 13 gene members, 5 of which are glutathione transferases (*GstE1, E3, E7, E8*, and *D1*) involved in response to oxidative stress and detoxification.

Class 5

Class 5 genes show immediate increase in expression levels reaching maximum at 0.5 h and later returned to the control levels at 2 h. This class identified 8 genes including a group of highly interesting genes involved in transcriptional regulation: *Sox14* is involved in the regulation of transcription, *tara* in chromatin-mediated maintenance of transcription, *elb* in RNA polymerase II transcription factor activity, *cbt* in positive regulation of transcription, *CG15678* in regulation of immune response. The remaining two genes, *CG15673* and *olf186-m* encode novel proteins. These two genes, although, encoding transcripts of unknown function may likely be involved or take part in the regulation of transcription. If they do, that would suggests that the class discovery method could as well be used in functional prediction which allows us to assign function to novel genes based on known functions of most of the genes constituting the gene classes. It would therefore be interesting to examine the biological function of these genes.

Class 6

Class 6 genes showed an early increase in expression level reaching a maximum at 1 h after which the expression levels fell gradually reaching the control levels at 4 h. Within this class, 8 genes were also discovered including a J-domain chaperone protein *DnaJ-1* and genes involved in zinc ion binding (e.g. *CG1600* and *CG6051).*

Class 7

Class 7 uncovered an up and down expression level patterns starting from 0.25 h with 6 genes contained in this class including those involved in transcriptional regulation (*psf2* and *CG13141*) and binding (*CG4408* and *CG3603*).

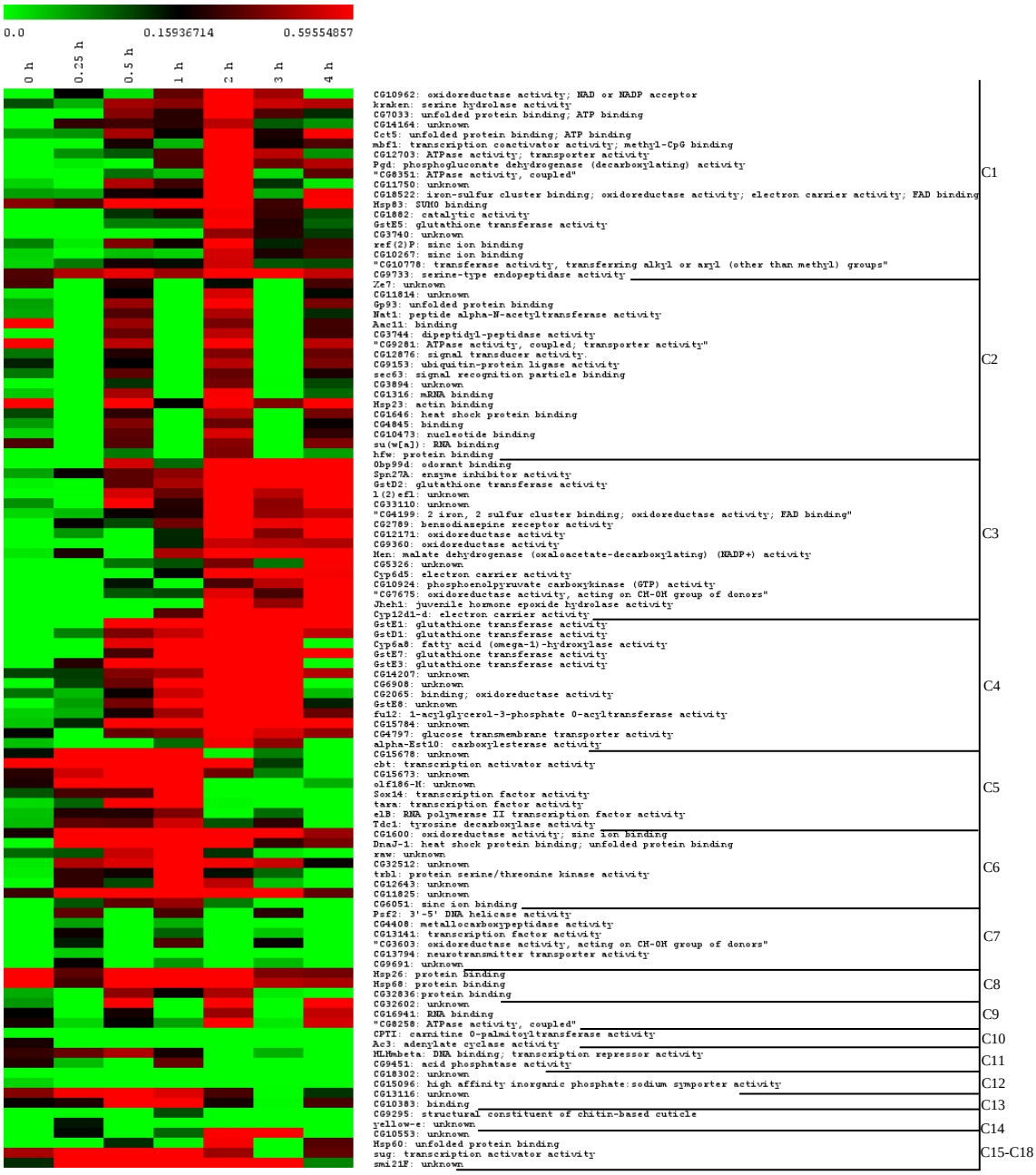
Class 8

Class 8 revealed an up and down transcriptional patterns from 0.5 h and the expression levels between 3 and 4 h showing level (constant) downregulation. In this class, we found three genes mainly *Hsp26, Hsp68* and *CG32836*, all of which are involved in protein binding.

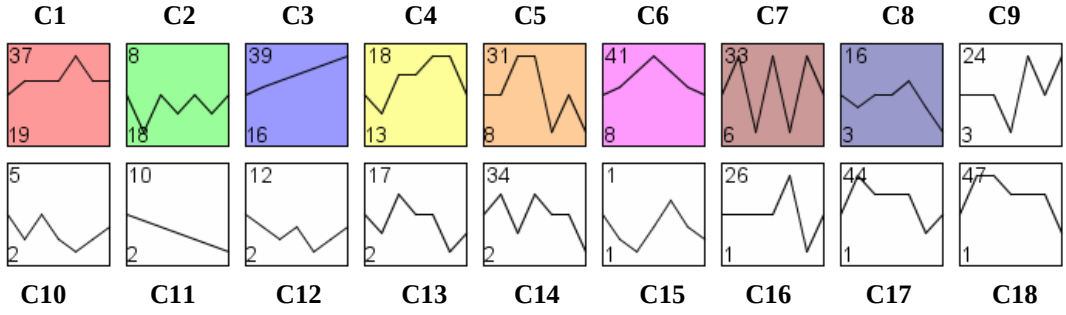
Interestingly, the majority of the transcriptional factors and heat shock proteins were upregulated as early as 0.25 h after acute ethanol exposure. The heat shock protein genes (see class 1, 2, and 8) had a unique up and down (bipolar) and early and late (biphasic) expression. Similarly, the downregulated gene classes (though not discussed) (see class 10, 12 and 14) also showed unique biphasic and bipolar patterns of gene expression. It is therefore interesting to note that of the 108 genes that were differentially expressed during 0 - 4 h after acute ethanol exposure, the most prominent ones were the genes showing both the up- and down-regulation trend. This may reflect genes specifically responsive to acute ethanol effect in the *Drosophila* head. Some of these genes include those encoding stress response, transcriptional regulation and proteolysis.

However, it should be noted that while the STEM clustering tool has been specifically designed for clustering short time series expression data like the one used in the current study, the results obtained using the software may prove difficult in deducing biological function from transcriptional response. This is because some of the profiles obtained from STEM are quite noisy and do not represent good clusters. This is quite evident on the heat map representation of these clusters shown in Figure 3.17 a.

(a)


0.0
0.15936714
0.59554857
0 h
0.25 h
0.5 h
1 h
2 h
3 h
4 h
CG10962: oxidoreductase activity; NAD or NADP acceptor
kraken: serine hydrolase activity
CG7033: unfolded protein binding; ATP binding
CG14164: unknown
Cct5: unfolded protein binding; ATP binding
mbf1: transcription coactivator activity; methyl-CpG binding
CG12703: ATPase activity; transporter activity
Pgd: phosphogluconate dehydrogenase (decarboxylating) activity
"CG8351: ATPase activity, coupled"
CG11750: unknown
CG18522: iron-sulfur cluster binding; oxidoreductase activity; electron carrier activity; FAD binding
Hsp83: SUMO binding
CG1882: catalytic activity
GstE5: glutathione transferase activity
CG3740: unknown
ref(2)P: zinc ion binding
CG10267: zinc ion binding
"CG10778: transferase activity, transferring alkyl or aryl (other than methyl) groups"
CG9733: serine-type endopeptidase activity
Ze7: unknown
CG11814: unknown
Gp93: unfolded protein binding
Nat1: peptide alpha-N-acetyltransferase activity
Aac11: binding
CG3744: dipeptidyl-peptidase activity
"CG9281: ATPase activity, coupled; transporter activity"
CG12876: signal transducer activity.
CG9153: ubiquitin-protein ligase activity
sec63: signal recognition particle binding
CG3894: unknown
CG1316: mRNA binding
Hsp23: actin binding
CG1646: heat shock protein binding
CG4845: binding
CG10473: nucleotide binding
su(w[a]): RNA binding
hfw: protein binding
Obp99d: odorant binding
Spn27A: enzyme inhibitor activity
GstD2: glutathione transferase activity
l(2)efl: unknown
CG33110: unknown
"CG4199: 2 iron, 2 sulfur cluster binding; oxidoreductase activity; FAD binding"
CG2789: benzodiazepine receptor activity
CG12171: oxidoreductase activity
CG9360: oxidoreductase activity
Men: malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity
CG5326: unknown
Cyp6d5: electron carrier activity
CG10924: phosphoenolpyruvate carboxykinase (GTP) activity
"CG7675: oxidoreductase activity, acting on CH-OH group of donors"
Jheh1: juvenile hormone epoxide hydrolase activity
Cyp12d1-d: electron carrier activity
GstE1: glutathione transferase activity
GstD1: glutathione transferase activity
Cyp6a8: fatty acid (omega-1)-hydroxylase activity
GstE7: glutathione transferase activity
GstE3: glutathione transferase activity
CG14207: unknown
CG6908: unknown
CG2065: binding; oxidoreductase activity
GstE8: unknown
fu12: 1-acylglycerol-3-phosphate O-acyltransferase activity
CG15784: unknown
CG4797: glucose transmembrane transporter activity
alpha-Est10: carboxylesterase activity
CG15678: unknown
cbt: transcription activator activity
CG15673: unknown
olf186-M: unknown
Sox14: transcription factor activity
tara: transcription factor activity
elB: RNA polymerase II transcription factor activity
Tdc1: tyrosine decarboxylase activity
CG1600: oxidoreductase activity; zinc ion binding
DnaJ-1: heat shock protein binding; unfolded protein binding
raw: unknown
CG32512: unknown
trbl: protein serine/threonine kinase activity
CG12643: unknown
CG11825: unknown
CG6051: zinc ion binding
Psf2: 3'-5' DNA helicase activity
CG4408: metallocarboxypeptidase activity
CG13141: transcription factor activity
"CG3603: oxidoreductase activity, acting on CH-OH group of donors"
CG13794: neurotransmitter transporter activity
CG9691: unknown
Hsp26: protein binding
Hsp68: protein binding
CG32836:protein binding
CG32602: unknown
CG16941: RNA binding
"CG8258: ATPase activity, coupled"
CPT1: carnitine O-palmitoyltransferase activity
Ac3: adenylate cyclase activity
HLHmbeta: DNA binding; transcription repressor activity
CG9451: acid phosphatase activity
CG18302: unknown
CG15096: high affinity inorganic phosphate:sodium symporter activity
CG13116: unknown
CG10383: binding
CG9295: structural constituent of chitin-based cuticle
yellow-e: unknown
CG10553: unknown
Hsp60: unfolded protein binding
sug: transcription activator activity
smi21F: unknown
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
C13
C14
C15-C18

(b)


C1
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17
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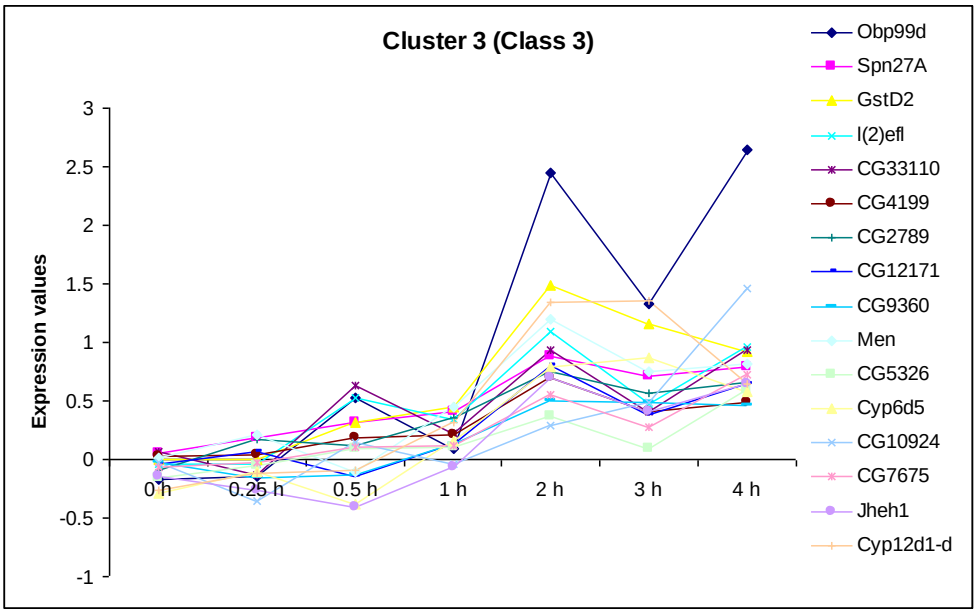
34
2
1
1
26
1
44
1
47
1
C10
C11
C12
C13
C14
C15
C16
C17
C18

Figure 3.17: Clustering of time-dependent changes in transcriptional response of *Drosophila* head to acute ethanol. (**a**) The figure depicts a heat map of the gene expression values containing colours (red, green and black) and molecular function of each gene represented. Each row represents the expression pattern of a single gene. The total expression pattern indicate genes identified in our microarray at a *P* value < 0.01 and shown on the right panel of the heat map. Each column represents one of the seven time points indicated. The colour represents the expression level of the gene. Red represents high expression, green represents low expression and black represents no change. The analysis was performed using the TM4 software (http://www.tm4.org). (**b**) Short Time-series Expression Miner (STEM) software (Ernst and Bar-Joseph, 2006) analysis divided the 108 genes into 18 clusters (i.e. 18 gene classes) designated C1…C18 shown on the heatmap. Each graph shows $\log_2$ expression values as a function of time for all genes in the heat map. The number at the upper and lower pane in each graph represent arbitrarily assigned model profile number and the number of genes in each cluster respectively. The colours represent significant clusters (classes) i.e. clusters containing higher number of genes than assigned to the model profiles. (**c**) The figure shows the profiles of all the 16 genes in STEM cluster 3 (designated C3 in STEM representative cluster).

(c)



3.3.7 Temporal Gene Expression Discussion

Here, a new picture of the gene expression profile in *Drosophila* head after being exposed to acute dose of ethanol in a time-dependent manner has been presented. This approach allowed exploration of specific classes of genes showing early or late responses to ethanol. It is conceivable that genes showing early responses to ethanol may depict immediate responses of *Drosophila* head to the acute effects of ethanol. Conversely, the late responsive genes may represent the physiological results from acute ethanol exposure because they were not expressed (induced or reduced) at early time points, but were altered at late time points. These ethanol responsive genes have therefore been classified into 8 significant clusters using STEM clustering tool.

Through the cluster analysis, it is conceivable that genes involved in mediating flies' tolerance to ethanol might be expected to remain up- or down-regulated at the end of 4 h time course. Similarly, genes with similar gene expression patterns and following similar time course may show similar response to ethanol sensitivity and or tolerance. However, some of these genes may not follow this pattern. For instance, cluster 3 (Figure 3.17 c) revealed a prominent gene, *malate enzyme* (*Men).* *Men* has been shown in transcriptional response to alcohol in *Drosophila* to be implicated in tolerance to ethanol (Morozova et al. 2006). It is however, possible that not all genes that affect tolerance are in cluster 3. In class 1, there are two genes experimentally validated in this study-*hsp83* and *mbf1*. These two genes affect both sensitivity and tolerance to ethanol (see Chapters 4 and 5). However, while *hsp83* showed decreased sensitivity and tolerance to ethanol, *mbf1* on the other hand showed increased sensitivity and decreased tolerance to ethanol. This may suggest that within this class, sub-classes exist and such affect sensitivity in different ways. However, for a meaningful conclusion to be drawn here, a validation test on other genes in the group is necessary. Class 1, 2, 3 and 4 have a large number of genes present. It is thought that each of these clusters may represent a unique functional label and be governed by the same regulatory elements or transcriptional factors binding sites. Again, this is yet to be validated. Thus, determining whether these putative classes produced by STEM clustering algorithm are meaningful requires further biological validation.

3.4 Chapter Discussion

In this chapter, a number of methods for analysing the microarray ethanol time course data set have been presented. Gene expression profiling coupled with various bioinformatics methodologies were used to examine transcriptional changes in *Drosophila* head exposed to acute ethanol.

Through the use of different statistical approaches and a combination of microarray data sets, an integrative search for putative genes regulated in response to ethanol-evoked changes in *Drosophila* head was carried out (Figure 3.5). The linear model for microarray analysis (LIMMA) implemented in BioConductor was implemented to detect differentially expressed genes, resulting in three different gene lists: a stringent list (P-0.05) used to query the fly base database and subsequently used in selecting genes of interests to be used in further work; a non- stringent list (P-0.1) used to depict patterns of enriched networks, signaling and metabolic pathways and finally a time-dependent list of regulated genes (time series list) used in class discovery. Results from all of these analyses culminate in a number of interesting findings: demonstrating the quantitative use of microarrays in the study of the genetic basis of alcohol disorder in the brain.

In order to select genes for biological validation and for future work, the P-0.05 list of genes which had passed through reasonably stringent filtering conditions, as directed by the need to control for problems often coupled with statistical analysis of microarray data (i.e. false positives), was employed. Filtering for viable alleles and establishing the availability of the selected genotypes (stocks) were also carried out. Thus, these criteria limited the choice of genes selected for behavioural validation using genetic methodologies.

On the time series analysis, testing for time-dependent changes in ethanol regulation in *Drosophila* head required that both the Sussex and Glasgow data sets be pooled and treated as a common dataset to allow joint analysis to be performed. There are many arguments for and against doing this. However, both arguments concluded that if pooling is necessary it must be carefully carried out so as not to obscure the biology underlying the study being investigated (see Morris et al.,

2006). In line with this, given that there were common reference controls to factor out any lab effect across the two labs, a decision to pool these data was made and a joint analysis performed on them.

Results from these analyses have shown that ethanol elicits profound and multiple changes or effects on different biological processes. In addition, candidate genes and gene networks that may play an important role in determining the behavioural responses to ethanol, as well possibly influencing addiction and or dependence have been identified. It was found that ethanol response in *Drosophila* is accompanied by reduction of transcripts levels for genes involved in metabolism, pigmentation, and transport activities. In contrasts, genes that encode detoxification and chaperone activities are up-regulated. Many other genes involved in transcriptional regulation, inter and intra cellular signaling, protein ubiquitination, neurogenesis, synaptic transmission, and circadian rhythm also appear to be responsive to ethanol. Many of these findings correlate with previous studies using *Drosophila* and other organisms.

Further, findings from alterations in gene sets (pathways) and networks have implicated a large number of metabolic pathways including glutathione metabolism, lindane degradation and fatty acid metabolism in *Drosophila* head exposed to acute alcohol. This highlights the potential importance of these pathways in mediating responses to alcohol.

In conclusion, this work shows that screening for ethanol-affected alterations in genes or gene sets (network and pathways) in *Drosophila* head reveals a large number of new candidate alcohol-regulated genes. These are in addition to the commonly found GABA-B and NMDA receptors genes. Furthermore, the data not only highlight potential new networks between these genes for understanding the biological basis of ethanol response, but also useful common pathway maps for further study. Finally, these types of analyses shed new light on the unique and common detoxification, signaling, stress and metabolic cascades underlying alcohol response and possibly, addiction.

Chapter 4.

Validating Candidate Genes for Ethanol Sensitivity.

4 Validating Candidates Genes for Ethanol Sensitivity

The analysis of gene expression (**Chapter 3**) following exposure to acute ethanol has uncovered some novel genes and pathways underpinning the genomic response to ethanol. The work described in this chapter describes experiments to validate some of these genes. The approach taken is to use flies carrying mutations in these genes in a series of behavioural tests. Section 4.1 provides a brief introduction to the study and discusses the need for validation and usefulness of mutant analysis, section 4.2 briefly mentions the experimental assays used and sections 4.3 to 4.5 presents an analysis of the results. Finally, section 4.6 summarises the conclusion of the investigation.

4.1 Introduction

As microarrays are essentially for screening purposes and the results obtained from them need to be validated, in this chapter, the 7 candidate genes of interest identified in the gene expression studies in chapter three were validated and linked to ethanol behavioural phenotypes using behavioural genetics approaches.

The fruit fly *Drosophila melanogaster* offers very powerful genetic tools with which to dissect genes and pathways underlying behavioural responses to ethanol (Heberlein et al., 2004; Berger et al., 2004; Berger et al., 2008). The fact that flies display many behaviours similar to acute intoxication in mammals when exposed to ethanol vapour (Heberlein 2000) coupled with the availability of powerful tools for genetic manipulation in *Drosophila* together with the high degree of conservation at the genomic level, make *Drosophila* a promising model organism to study the mechanism by which ethanol regulates behaviour.

Many investigators have, therefore, begun to determine ethanol sensitivity in *Drosophila* by using mutations in genes suspected to be involved in the ethanol response (Moore et al., 1998; Berger et al., 2008; Wen et al., 2005). For example, the mutant *neuropeptide F* (*NPF*, a homolog of the mammalian neuropeptide Y) flies and its receptor NPFR-1 was shown to be involved in decreased ethanol sensitivity compared with wild-type flies (Wen et al., 2005); the *cheapdate* allele of *amnesiac* (Feany and Quinn, 1995) encoding a neuropeptide thought to activate the cyclic

AMP signaling pathway was implicated in increased ethanol sensitivity (Moore et al., 1998); mutations in the *rutabaga* (*rut*) gene encoding a calcium/calmodulin-dependent adenylate cyclase displayed increased ethanol sensitivity(Moore et al., 1998) as does *Fasciclin II* (*Fas2*) (Cheng et al., 2001); while mutation of the *PKa-RII*, which encodes a cyclic AMP-dependent protein kinase, caused a reduced sensitivity to ethanol (Park et al., 2000).

Mutant analyses have been previously used to validate genes identified from microarray studies (Morozova et al., 2006). One was an excellent study by Morozova et al., (2006) that investigated transcriptional changes in *Drosophila* following acute alcohol exposure. The study analysed 20 genes out of which 15 were implicated in the ethanol response in *Drosophila*. Thus, behavioural screening for genes with altered ethanol response from the microarray list of differential expression is an excellent approach to identify novel genes mediating alcohol-induced behaviours.

The goal of this chapter therefore is to link candidate genes obtained from the gene expression analysis to ethanol behaviour using behavioural genetic methodologies. This is a powerful method to determine the degree of association between a phenotype and a gene (Crabbe et al 1983). This approach also has the advantage that it can reveal different aspects of gene function that cannot be revealed by non-genetic approaches.

4.2 Methods

To validate the microarray results as well as to determine whether the transcriptional changes observed were specific to the ethanol-responsive genes, three different paradigms namely, inebriometer, sedation and recovery assays were used (see section of the Materials and Methods). However, as will be seen later, only sedation and recovery assays were later used throughout the experiment. All of these assays have previously been used to examine the fly's response to ethanol.

Ethanol vapour has been used as an effective method to deliver a reproducible ethanol dose and to rapidly sedate flies (Moore et al., 1998; Wen et al., 2005;

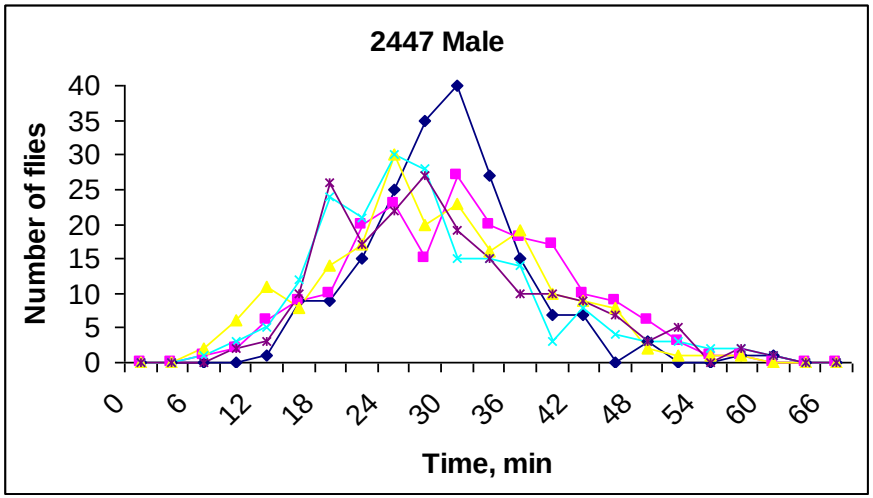
Cowmeadow et al., 2005). When flies are exposed to ethanol vapour, they first enter a hyperactive state in which they display fast walking and flying activities. This overt increase in their hyperkinetic activities only lasts for a few minutes after which their movement gradually subsides and later stops, thus reaching a point at which they eventually become sedated.

4.3 Inebriometer

The sensitivity of a population of flies to ethanol can be measured in the inebriometer, an apparatus that quantifies ethanol-induced loss of postural control (Heberlein, 2000, Hancock, 2005). Two strains of Oregon R fly stocks named 2447 and 2451 isogenised on the second and third chromosomes (Sharma et al., 2005; section 2.3.2 of Materials and Methods) were tested in the inebriometer. To determine if flies are sensitive to the inebriating effects of ethanol, they were introduced into the inebriometer at a standard ethanol/air mixture (see section 2.3.1 of Materials and Methods). Flies from each strain were separately put into the top of the inebriometer and the number that eluted every 3 minutes was recorded. At least five repeats of the single exposure were completed in the inebriometer runs for both the 2447 and 2451 (Figure 4.1) wild-type controls to give a measure of within strain error**.** This assay was first carried out to determine which of the two strains was suitable and might be valuable for use as the general wild-type control in further behavioural testing and to establish the standard laboratory mean elution time (MET) for other testing.

Comparing all the METs for both male and female strains using a one-way ANOVA showed a marginal effect towards the strains ($F_{3,14}$ = 3.55, P = 0.04). Significant difference was seen between 2447 male and 2447 female; 2447 male and 2451 female, $P < 0.001$. However, none of 2447 male vs. 2451 male and 2451 male vs. 2451 female showed sufficiently different postural control under the ethanol exposure to be considered significantly variable $P > 0.05$. In all, however, the 2447 strain show a tighter and compact profile as indicated by its standard error (0.49) compared to 2451. The 2447 strain was therefore chosen as the wild-type strain and used in the subsequent behavioural analysis described in this thesis.

A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Number of flies
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Time, min

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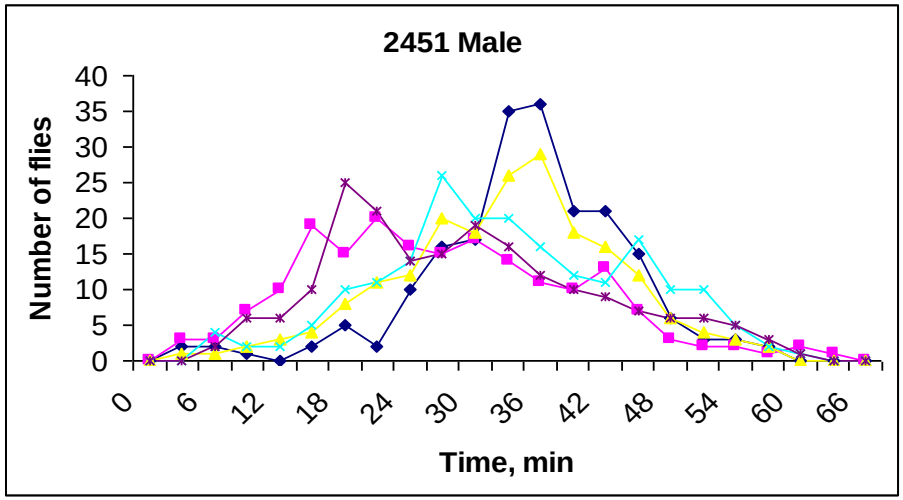
2451 Male
Number of flies
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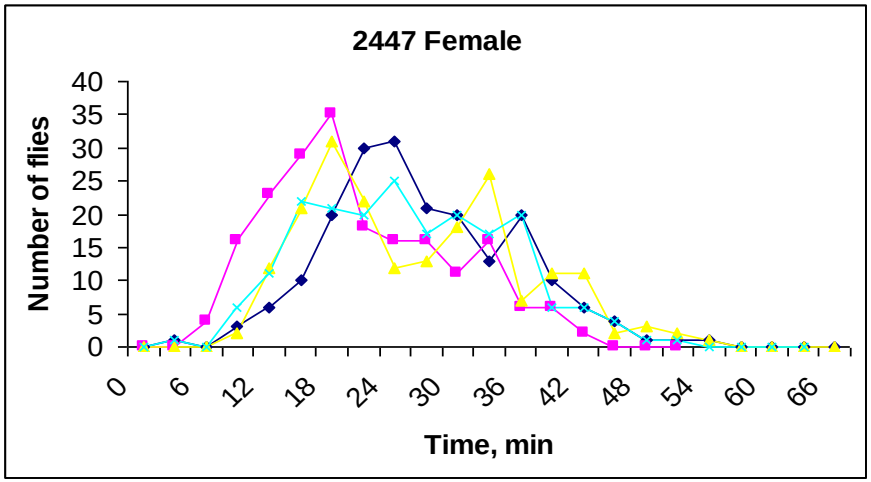
2447 Female
Number of flies
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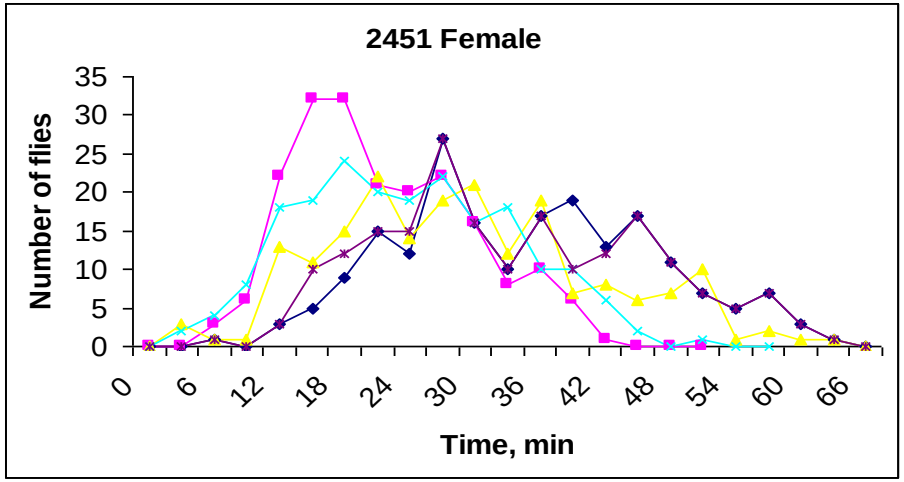
2451 Female
Number of flies
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60
66
Time, min

E

Genotype	MET ± SEM (min)	n
2451 Male	31.42±1.4	5
2451 Female	27.29±2.96	4
2447 Male	28.46±0.45	5
2447 Female	24.48±1.74	4

F

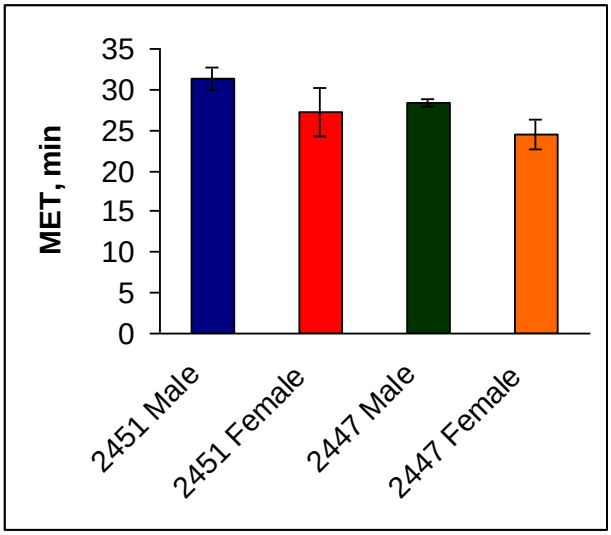


Figure 4.1| Ethanol sensitivity of the *Drosophila* isogenic wild-type strains. (A, B, C and D). Individual inebriometer elution profiles for the 4 isogenic male and female genotypes. All runs started and ended between 8.30 am and 12 pm. All completed runs in each of the four individual profiles show similar trends in the curve shape and attributes. A One-way ANOVA analysis of the data set specific for each individual profiles of A-D shows there are no significant differences among them ($P > 0.1$). In all, the profiles of 2447 male appear tighter and more compact than others in the comparison. (E) Average mean elution times (MET) of the isogenic lines. (F) Bar graph represents inebriometer METs (±SEM) of the 4 isogenic genotypes tested for ethanol sensitivity showing that 2447 male has the lowest SEM (i.e. the lowest behavioural variations). The 2447 genotype was therefore used as the general wild-type control for other analyses; here and elsewhere in this chapter n represents the number of experiments, not the number of flies; in all figures error bars represent standard errors of the mean.

4.3.1 Inebriometer Discussion

The inebriometer has been described as offering an established and well known behaviour assay, which investigates a population of flies thus producing reduced error rates in the data (Hancock, 2005). Testing the isogenic lines 2447 and 2451 showed no significant variation between populations. The two strains therefore offer suitable choice for use as wild type control flies. However, for the purpose of further behavioural testing, it is required that one strain be selected and the choice of 2447 was determined based on the inebriometer profiles and within flies error rates results as previously described.

Further, it should be noted that most important consideration in this study is to find a robust, reproducible technique that is sensitive to small variations in ethanol sensitivity. Thus, while the inebriometer offers a good means for measuring the sensitivity of *Drosophila* to ethanol (Heberlein, 2000), the assay is highly sensitive to variables including number of flies, the time of the assay and the temperature meaning that this may not offer much needed robust data. In addition, it is also very tedious to use it where large numbers of repeated exposures are required.
Finally, the inebriometer assay was therefore not used beyond this point.

4.4 Sedation and Recovery Assays

The inebriometer measures the flies' postural control after ethanol exposure (Moore et al., 1998; Singh and Heberlein, 2000). Two different behavioural methods were first published by Wen et al (2005) to measure sensitivity to ethanol exposure in *Drosophila*. The sedation assay measures the duration that a fly remains active when faced with an ethanol stress before becoming sedated, while the recovery assay measures the rate of recovery from a sedating dose of ethanol (see section 2.3 of the Materials and Methods for a detailed description of the assays). These two assays were modified and used for testing the candidate genes' responses to ethanol. The fraction of flies that became sedated or recover was measured as a function of time (Figures 4.2 and 4.3).

4.4.1 Sedation assay measures the duration of activity

To determine the most appropriate dose of ethanol to use in the sedation assay, an ethanol-dose response test was performed using the 2447 wild type stock (designated, Ctl). Ethanol solutions of 10%, 30%, 40%, 50% and 80% were made by mixing ethanol and water at the ratio (vol/vol) of 1:9, 3:7, 4:6 and 8:2 respectively and used to measure the flies resistance to the sedative effect of ethanol as described in section 2.3 of the Materials and Methods. Flies displayed behavioural changes when exposed to the appropriate dose of ethanol: they became hyperactive, lost motor control and eventually became sedated. At the end of the experiment the effect of ethanol dose and duration were evaluated. Flies exposed to 100% EtOH vapour showed a mean sedation time (MST) of 20.5±0.2 min whereas it took longer

with 80% EtOH (MST 32.3±0.5 min), 50% EtOH (MST 53.3±0.5 min) and 40% EtOH (MST 61.7±0.5 min) (Figure 4.2).

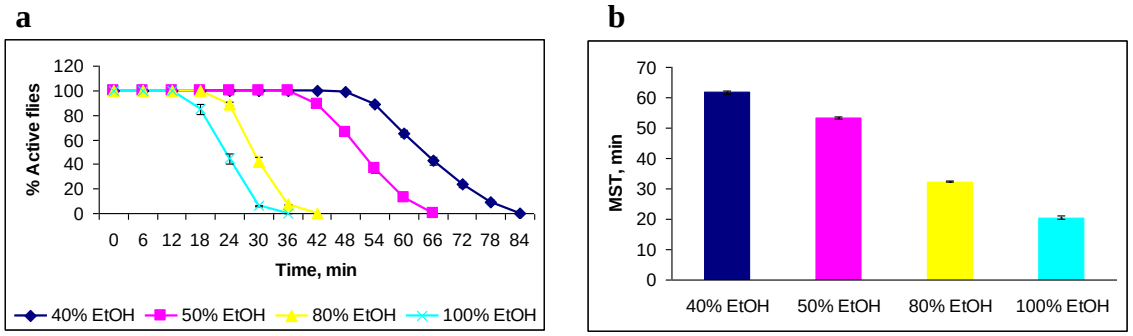


Figure 4.2 | Effects of ethanol dose on male wild-type 2447 (Ctl) flies.
(a)The duration of activities of flies undergoing sedation correlates with ethanol dose of 40%, 50%, 80% and 100% as indicated. For these and all subsequent sedation curve data, the percentage of flies scored as active during sedation in a group of 20 male flies is graphed as a function of time. (b) Mean sedation time (MST) for experiment represented in (a) a one-way ANOVA analysis revealed significant main effects of ethanol dose across the various concentrations used (P<0.0001). Error bars represent the SEM (n=20 in all EtOH concentrations); n corresponds to the number of experiments, not the number of flies.

4.4.2 Dose response Discussion

For the dose response study in the sedation assay, the data for 10 % and 30% EtOH vapour concentrations were not included as it took a very long time for the flies exposed to these concentrations to became sedated. As expected, sedation was dose dependent, such that lower ethanol concentration resulted in longer MSTs (Figures 4.2). In order to choose a suitable exposure concentration, it was necessary to compare all the METs and profiles of the percentage active flies at the end of the sedation experiment at all concentrations (excluding 10 and 30% EtOH concentrations) to allow a clear decision to be made. A 100% dose gives a sharp, clear peak of activity but has a short lived reaction, suggesting that any alterations in sensitivity might be difficult to quantify because of rapid immobilisation and the fact that these concentrations may be deleterious to the flies. At 50% EtOH concentration, it takes a reasonable amount of time to sedate the flies and it is expected (based on the profiles) that small differences in sedation time could be detected at this concentration. A 40% EtOH concentration also offers alterations in

sensitivity that might easily be measured but the MST is rather long. Thus, 50% EtOH concentration was chosen and used in the subsequent sedation protocol described in this thesis.

4.4.3 Recovery assay measures the duration of intoxication

The recovery assay quantifies the behaviour of flies as they recover from the intoxicating dose of ethanol. Specifically, it measures the recovery of flies' activities as indicated by their flying, walking and innate climbing behaviours in the vial immediately after they were exposed to a sedating dose of ethanol for a defined period of time (see section 2.2.4 of the Materials and Methods for a detailed description of the assay). The fraction of flies that had recovered from intoxication was measured as a function of time (Figure 4.3).

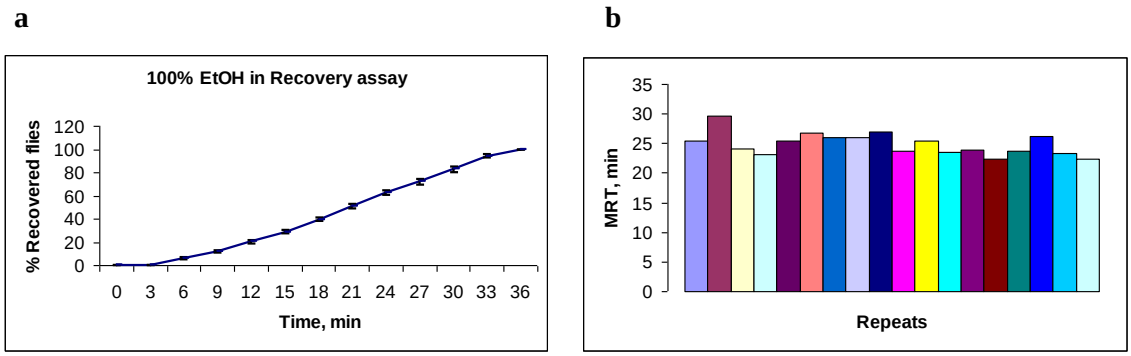


Figure 4.3 | Recovery from intoxication of normal ethanol-sensitivity of 2447 wild-type flies (Ctl). (a) Recovery curves and (b) MRT for the wild-type control flies. One-way ANOVA revealed no significant difference within repeats. n=18 and represents the number of experiments, not the number of flies; Error bars represent the SEM.

4.5 Ethanol Sensitivity of Mutant Flies

To measure sensitivity of the 7 candidate genes to the sedative effects of ethanol, each line was assayed in sedation and recovery paradigms (Wen et al., 2004). When mutant alleles of the 7 genes (excluding controls) were tested, the resulting MSTs in the sedation assay ranged from a low of 39.4 ± 0.3 minutes (hiw^{EP1308}) to a high of 78.6±1.0 minutes (hop^{27}/+) (Figure 4.4 a, Table 4.1). Similarly, when these mutants were tested in the recovery assay, the resulting MRTs ranged from a low of 10.0 ± 0.2 minutes ($hsp83^{08445}$) to a high of 40.79 ± 0.9 minutes ($mbf1^{2}$) (Figure 4.4 b, Table 4.1).

All the ethanol sensitivity data were compared by a student's paired t-test assuming equal variance. This approach enhanced the statistical sensitivity of the analysis and ensured that mutants with alterations in behaviour were not being missed when compared to the wild type control.

In summary, 10 mutant alleles (out of 12 alleles) corresponding to 6 genes showed significant alterations in ethanol sensitivity in the sedation assay. In this assay, 4 mutant alleles corresponding to 2 mutant genes ($mbf1^{2}$, hiw^{EP1305}, hiw^{EP1308} and hiw^{ND8}) showed enhanced ethanol sensitivity while the remaining 6 alleles corresponding to 4 genes (hop^{25}/+, hop^{27}/+, $hsp83^{08445}$, $hsp83^{e6A}$/+, ana^{1} and $Axn^{EY10228}$/+) displayed reduced sensitivity to ethanol. Similarly, when the 12 mutants were tested in the recovery assay, they all showed altered ethanol sensitivity. In this assay, 4 ethanol-sensitive mutants- $mbf1^{2}$, hiw^{EP1305}, hiw^{EP1308} and hiw^{ND8} recovered more slowly (higher MRTs) than wild-type flies (Table 4.1 and Figure 4.4). Conversely, the ethanol-resistant mutants hop^{25}/+, hop^{27}/+, $hsp83^{08445}$, $hsp83^{e6A}$/+, ana^{1} and $Axn^{EY10228}$/+ recovered more quickly, with lower MRTs (Table 4.1 and Figure 4.4). Therefore, these 10 mutants show a recovery assay phenotype that is consistent with their sedation assay phenotype. However, 2 mutants- $hsp26^{EY10556}$ and $hsp26^{KG02786}$, which did not exhibit significant difference in sedation assay MST compared with wild-type control (Table 4.1 and Figure 4.4) showed reduced ethanol sensitivity as they recovered significantly faster than wild-type flies. The ethanol resistant phenotype of the two *hsp26* mutants only in the recovery assay thus define a genetic pathway involved specifically in the recovery of *hsp26* deficient flies from ethanol exposure.

It should be noted that to verify the above results and ensure that these phenotypes were actually due to ethanol response and not as a result of the flies' genetic background, two approaches were adopted. First, screening for more alleles of the same stocks was carried out. This allowed responses that could easily be seen on a variety of alleles of the same stocks to be studied thereby ruling out any effect due to background. Second, where it could not be possible to screen for more alleles, additional 'control' flies were generated from the original stocks and used in assessing any effect due to genetic background. These confirmatory analyses are explained in the various sections for the individual genes below.

Table 4.1 | Ethanol Sensitivity of the Candidate Genes.

Gene	Mutant	MST1, min. ±SEM, (n)	MST1, min. ±SEM, (n)	Special notes on the genotypes used
Ctl		53.3 ± 0.3 (20)	24.9 ± 0.4 (18)	wild-type control genotype (male)
Ctl (F)		52.6 ± 0.7 (6)	21.7 ± 0.5 (6)	wild-type control genotype (female)
ana^{1}	*ana*^{1}	72.7 ± 0.9 (6); R	13.6 ± 0.1 (6); R	
	ana^{1}*/Cyo*	80.1 ± 1.4 (9); R	11.4 ± 0.1 (6); R	
	P[ana+]; ana^{1}	60.7 ± 0.3 (6); R	20.4 ± 0.3 (6); R	Genotype carrying *ana*^{1} rescue construct and used as normal control
Axin	*Axn* ^{EY10228}*/+*	66.9 ± 0.2 (6); R	16.5 ± 0.2 (6); R	Generated from a cross between *axn* $^{EY10228/}$ *TM3* female and Ctl male flies
	Axn^{EY10228}*/TM3*	61.6 ± 0.3 (6); R	16.8 ± 0.1 (6); R	Original stock; recessive lethal
	TM3/+	70.3 ± 0.2 (6); R	13.7 ± 0.4 (6); R	Generated from a cross between *axn* $^{EY10228/}$ *TM3* female and Ctl male flies; control
Hiw	*hiw*^{EP1308}	39.5 ± 0.3 (8); S	33.1 ± 0.2 (8); S	
	hiw^{EP1308} *(F)*	39.8 ± 0.2 (6); S	28.4 ± 0.3 (6); S	
	hiw^{EP1308}*/Y*	49.8 ± 0.2 (6); S	23.3 ± 0.2 (6)	Generated from a cross between homozygous female hiw^{EP1308} and Ctl male flies
	hiw^{EP1305}	40.3 ± 0.2 (6); S	29.1 ± 0.2 (6); S	
	hiw^{EP1305} *(F)*	40.3 ± 0.2 (6); S	26.9 ± 0.2 (6); S	
	hiw^{ND8}	41.8 ± 0.3 (6); S	38.0 ± 0.5 (6); S	
	hiw^{ND8} *(F)*	41.1 ± 0.2 (6); S	33.8 ± 0.1 (6); S	
hop	*hop*^{25}*/+ (F)*	78.3 ± 1.1 (6); R	11.4 ± 0.2 (6); R	Generated from a cross between *hop*^{25}*/Basc* female and Ctl male flies
	hop^{27}*/+ (F)*	78.6 ± 1.0 (6); R	12.5 ± 0.4 (6); R	Generated from a cross between *hop*^{27}*/Basc* female and Ctl male flies
	Basc/+ (F)	54.6 ± 0.6 (6)	20.3 ± 0.4 (6)	Generated from a cross between *hop*^{27}*/Basc* female and Ctl male flies; control
Hsp26	*hsp26*^{EY10556}	54.0 ± 0.2 (6)	22.6 ± 0.2 (6); R	
	hsp26^{EY10556}*/+*	53.1 ± 0.2 (6)	21.9 ± 0.2 (6); R	Generated from a cross between homozygous female *hsp26*^{EY10556} and Ctl male flies
	hsp26^{EY10556} *(F)*	62.9 ± 0.4 (8)	–	
	hsp26^{KG02786}	53.1 ± 0.4 (6)	21.3 ± 0.2 (6); R	

Gene	Mutant	MST1, min. ±SEM, (n)	MST1, min. ±SEM, (n)	Special notes on the genotypes used
Hsp83	*hsp83^{08445} (F)*	74.5 ± 2.3 (8); R	10.0 ± 0.1 (6); R	Obtained from original stock; female fertile, male sterile
	hsp83^{08445} / TM3(F)	50.1 ± 0.6 (6)	22.3 ± 0.4 (6)	Original stock, behaved like wild-type and used as normal control
	hsp83^{e6A} / + (F)	63.4 ± 0.3 (6); R	14.9 ± 0.4 (6); R	Generated from a cross between *hsp83^{e6A} / TM6B* female and Ctl male flies
	TM6B / + (F)	53.9 ± 0.3 (6)	20.5 ± 0.2 (6)	Generated from a cross between *hsp83^{e6A} / TM6B* female and Ctl male flies; control
mbf1	*mbf1^{2}*	39.5 ± 0.7 (6); S	40.8 ± 0.9 (6); S	
	mbf1^{+}	51.2 ± 0.6 (6)	22.1 ± 0.2 (6);	Genotype carrying *mbf1^{2}* rescue construct and used as normal control

Gene annotations were based on Flybase (http://flybase.bio.indiana.edu). Ethanol sensitivity was quantified in the sedation and recovery assays (see section of Materials and Methods). All values are mean ±SEM. For each genotype, n= number of experiments and not the number of flies. The behavioural control (Ctl) used consisted of a strain isogenised on the second and third chromosomes, *w+; Iso2C; Iso3I* (Sharma et al., 2005). This strain has been reported to behave like wild-type in a range of behavioural tests (Sharma et al., 2005). F (female flies) were used and where not specified male flies were used; S and R indicate lines that are significantly more sensitive and more resistant than the controls used.

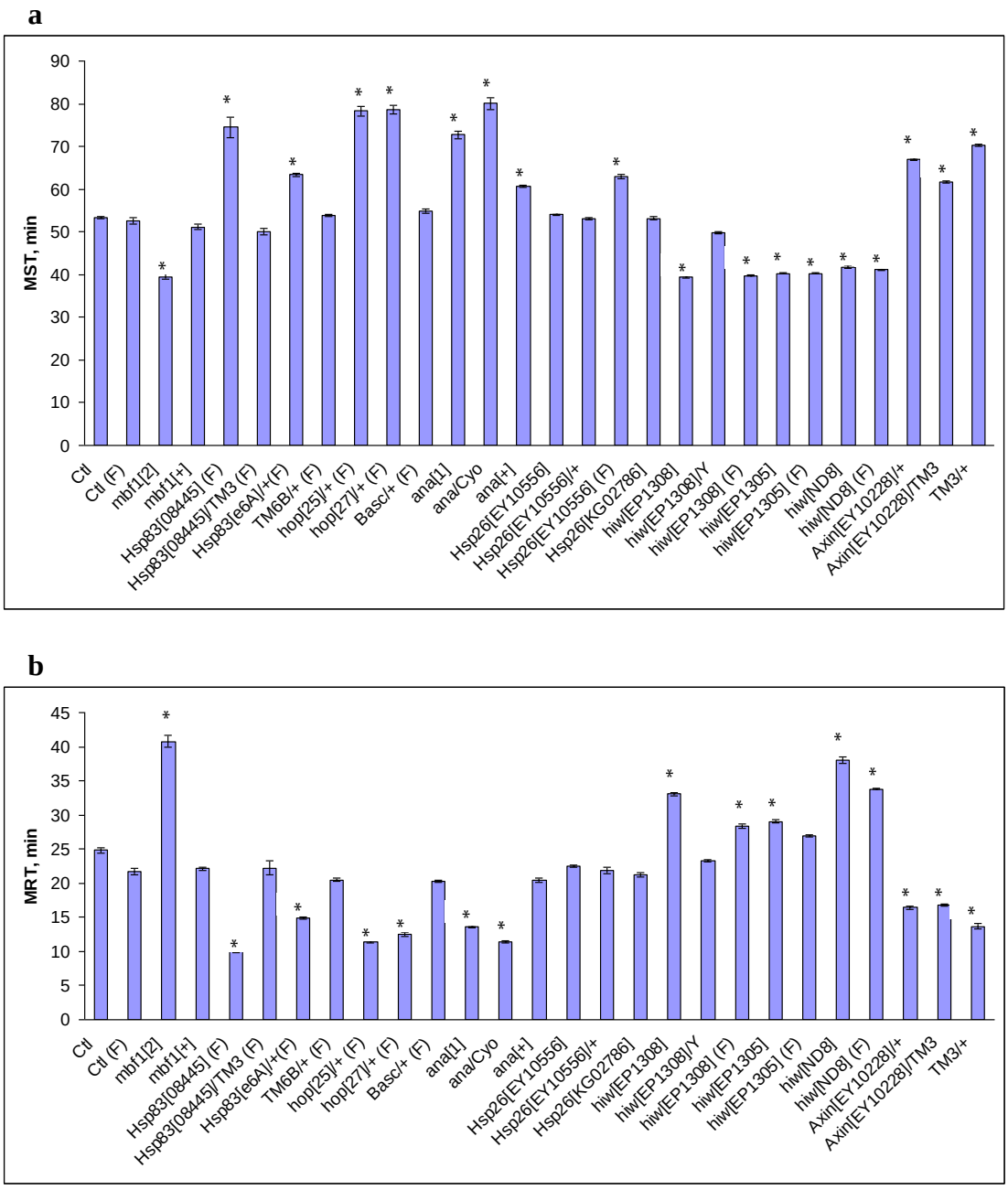


Figure 4.4 | Ethanol sensitivity of the candidate genes.

(a) Bar graph represents sedation MSTs of the mutants showing altered ethanol sensitivity (Ctl, control; *denotes $P < 0.0001$ for each mutant vs. Ctl). (b) Recovery MRTs of the mutants showing altered ethanol sensitivity ($^{**}P < 0.001$ for each mutant vs. Ctl). n=6-20 and represents the number of experiments, not the number of flies; error bars represent standard errors of the mean.

4.5.1 *ana* and Ethanol Sensitivity

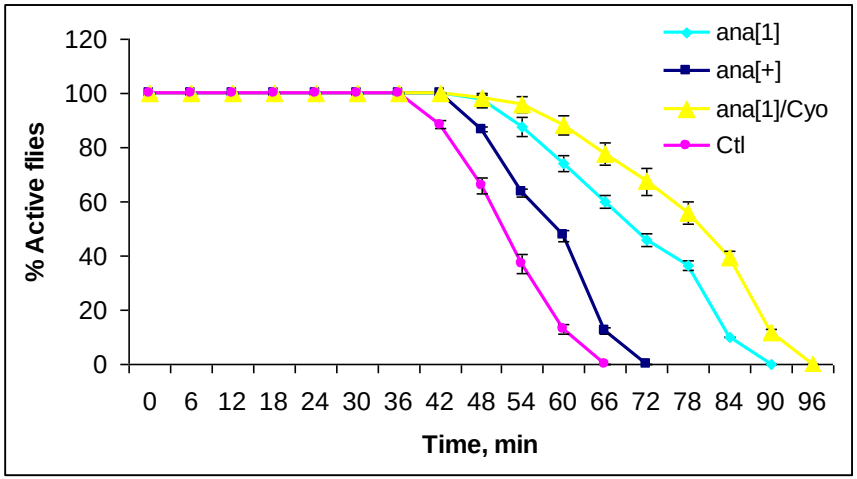
Drosophila anachronism gene is a secreted glia glycoprotein that prevents premature neuroblast proliferation. The ana allele ana^{1} tested in the sedation and recovery assay has been previously described (Ebens et al., 1993; Park et al., 1997). ana^{1} mutation is caused by a *P* element insertion in the fourth intron of ana^{1}. It is a strong partial-loss- of function allele caused by the insertion of a *P* element carrying the lacZ gene. An ana^{1} stock homozygous for both ana^{1} and a rescue construct P[ana^{+}] inserted on the first chromosome called P[ana^{+}]; ana^{1}, was used as a normal control thereby minimising the effects of genetic background.

It was observed that flies homozygous for ana^{1}displayed a statistically significant decrease in ethanol sensitivity compared with P[ana^{+}]; ana^{1} and wild-type controls ($P < 0.0001$; Figure 4.5). Similarly, these flies homozygous for ana^{1} recovered significantly faster than P[ana^{+}]; ana^{1} and wild-type controls ($P < 0.0001$; Figure 4.4). The heterozygous ana^{1}/CyO mutants also exhibited this reduced sensitivity to ethanol sedation; however, the CyO chromosome seems to be showing clear ethanol phenotype ($P < 0.0001$; Figure 4.4). The Cyo chromosome in this case enhanced the reducing ethanol sensitivity effect of the *ana* gene suggesting that Cyo is additive to the ethanol resistance causing ana^{1} allele. In addition, flies carrying the P[ana^{+}]; ana^{1} construct also displayed significantly reduced sensitivity to ethanol when compared with the wild-type control. Two phenomena may in part explain the behaviour seen with the *ana* rescue line: First, the sensitivity could be due to positional effect; that is the *P* element in P[ana^{+}]; ana^{1} inserted into a region that is not particularly transcriptionally active, in which case the *ana* gene would not be transcribed at a level required to restore normal sensitivity to ethanol. Second, it is possible that the design of the *ana* cDNA did not take into account the precise transcription that is critical for normal ethanol sensitivity; that is the promoter region required to properly initiate full transcription of the *ana gene* was not fully accessible to the *ana* cDNA in the *P* element rescue construct and could therefore not transcribe the gene at a level required to restore normal ethanol sensitivity - a behavioural phenotype that is very sensitive to the level of transcription. Regardless of the exact reason, the data clearly show that mutation affecting the *ana* gene lead to decreased ethanol sensitivity.

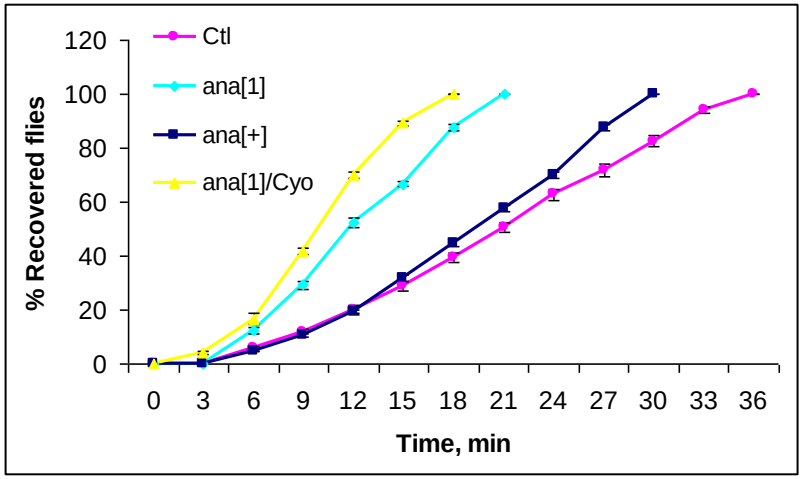
Figure 4.5 | *ana*^{1} flies have reduced ethanol sensitivity.

(a). Left panel shows the mean sedation profiles for flies exposed to ethanol. **(b)** Shows the recovery time from ethanol intoxication of these flies. In **(a** and **b)** *ana*^{1} mutants (*ana*^{1} and *ana1/CyO*) show reduced ethanol sensitivity compared with two controls, (Ctl) and *P*[ana+]; ana^{1} (designated as ana[+] in the graph). **(c and d)** Mean sedation time (MST) and mean recovery time measuring the flies' resistance to and recovering from the sedative effects of ethanol respectively. *ana*^{1} mutants show significantly reduced ethanol sensitivity compared with two controls, (Ctl) and ana[+] . $^{*}P < 0.0001$ in **(c)**; $^{*}P < 0.001$, $^{**}P < 0.0001$ in **(d);** n=6-20 experiments. In all panels error bars represent SEM.

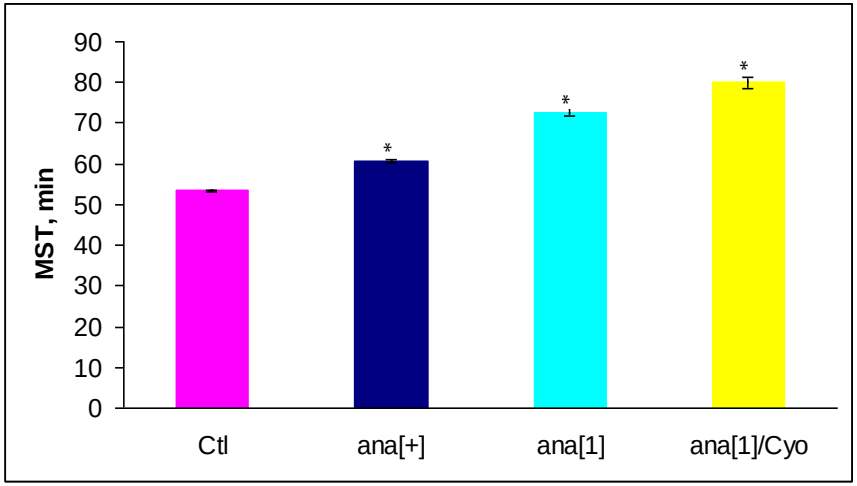
a


% Active flies
Time, min
ana[1]
ana[+]
ana[1]/Cyo
Ctl

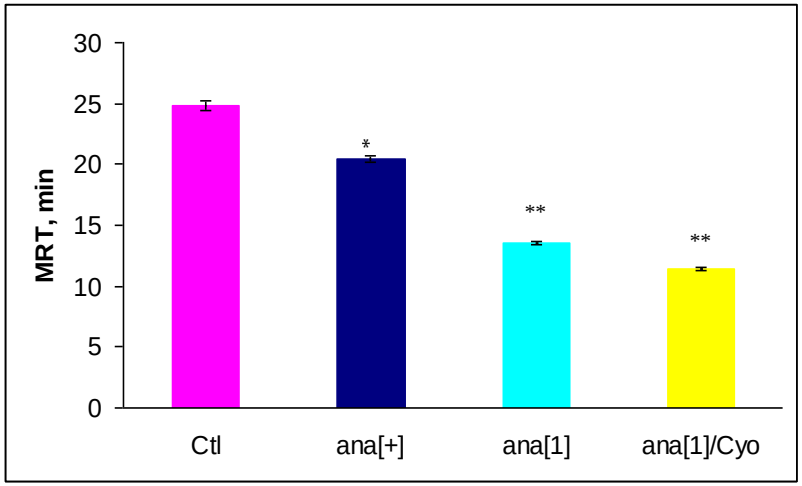
b


% Recovered flies
Time, min
Ctl
ana[1]
ana[+]
ana[1]/Cyo

c


MST, min
Ctl
ana[+]
ana[1]
ana[1]/Cyo

d

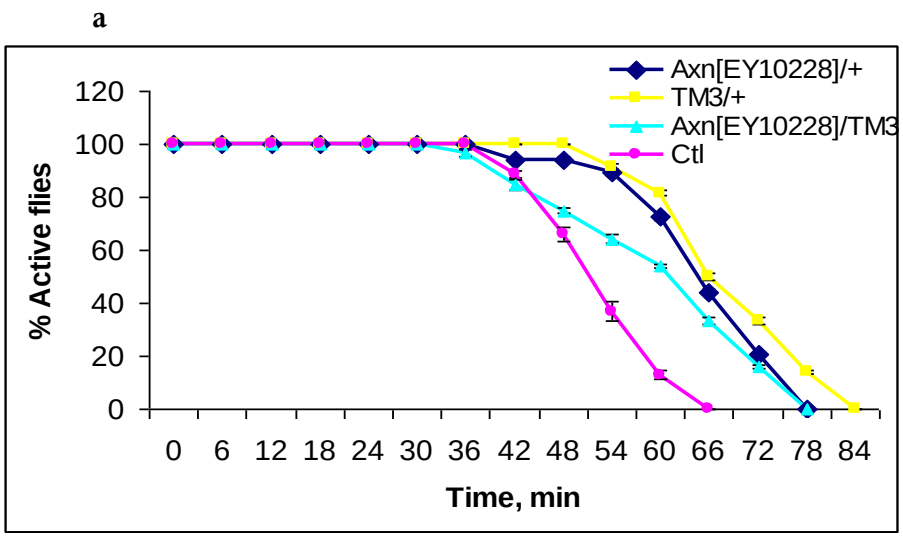

MRT, min
Ctl
ana[+]
ana[1]
ana[1]/Cyo

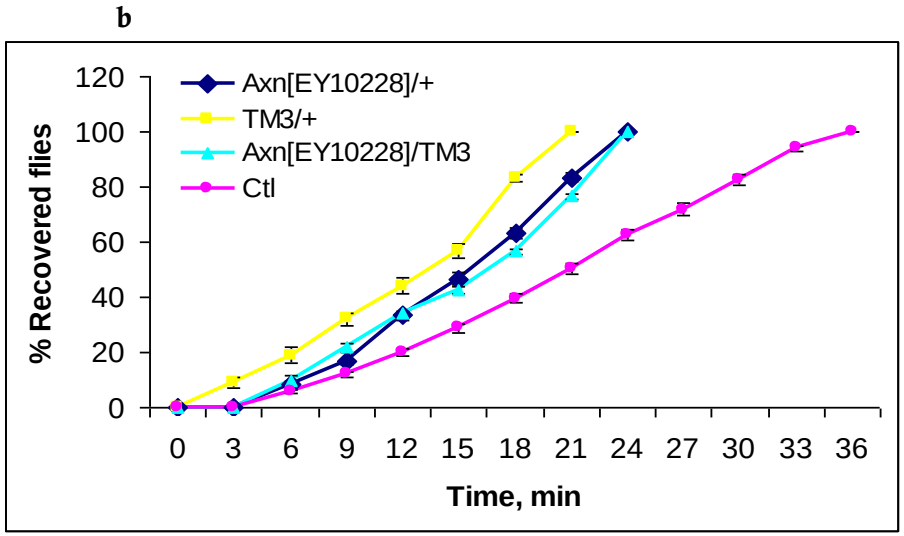
4.5.2 *Axn* and Ethanol Sensitivity

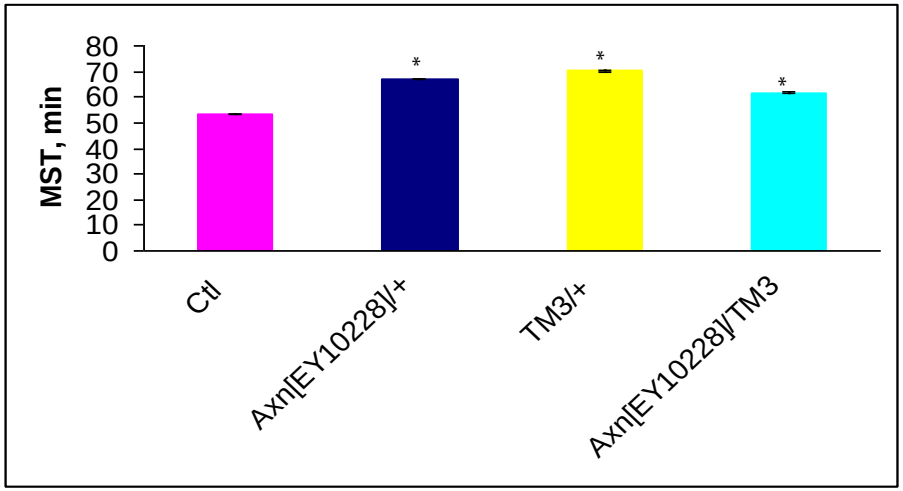
Drosophila Axin gene is a negative regulator of the wingless signaling pathway. To determine whether the *Axn* gene is implicated in ethanol sensitivity, the recessive lethal mutant $Axn^{EY10228}$ was assayed in the sedation and recovery paradigms. Male flies from this mutant balanced over the third multiple balancer chromosome, TM3 was crossed with homozygous virgin wild-type female controls to generate two distinct genotypes: $Axn^{EY10228}$/+ and *TM3*/+ flies. These three lines *Axn*/*TM3* (original stock), *Axn*/+ and *TM3*/+ were then tested for ethanol sensitivity in the two assays. *Axn*/*TM3*, *Axn*/+ and *TM3*/+ flies all displayed a statistically significant decrease in ethanol sensitivity compared with wild-type control ($P < 0.0001$; Figure 4.6). However, as can be seen in Figure 4.5 both *Axn*/+ and TM3/+ displayed a reduced ethanol sensitivity higher than heterozygous combination of axn/TM3 in the sedation assay. One possible explanation for this is that *Axn* and *TM3* may be involved in the same ethanol sensitivity pathway in a partial compensatory manner. Nevertheless, one cannot rule out the effect due to genetic background, it is clearly seen that *Axn* causes a decrease in ethanol sensitivity because the reduced ethanol sensitivities are different in both *Axn* and *TM3* flies (clearly evident in Table 4.1).

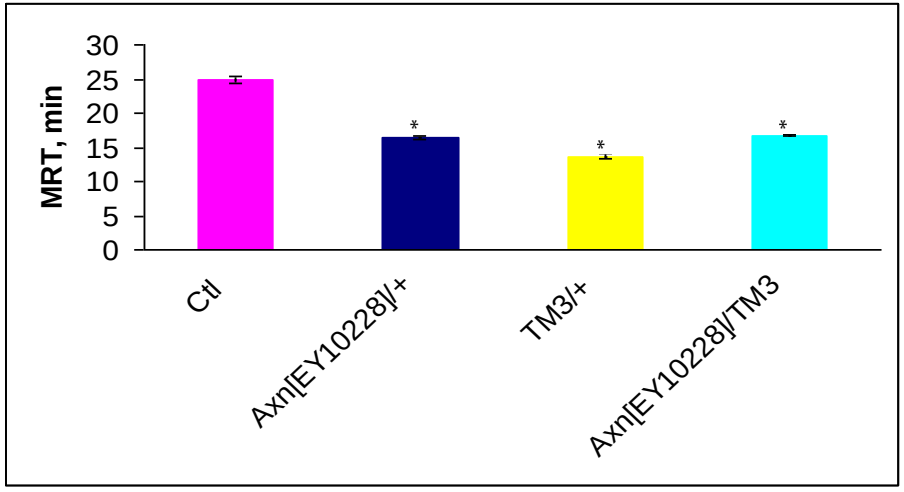
Figure 4.6 | *Axn*^{EY10228}/+ flies have reduced ethanol sensitivity.

(a) Left panel shows the mean sedation profiles for flies exposed to ethanol. **(b)** Shows the recovery time from ethanol intoxication of these flies. In **(c and d)** *Axn*^{EY10228}/+ mutants show reduced ethanol sensitivity compared with appropriate controls, Ctl and *Axn*^{EY10228}*/TM3*. **(c** and **d)** Mean sedation time (MST) and mean recovery time measuring the flies' resistance to and recovering from the sedative effects of ethanol respectively. *Axn*^{EY10228}/+ mutants show significantly reduced ethanol sensitivity compared with the controls. $^{*}P < 0.0001$**;** n=6-20 experiments. In all panels errors bars represent SEM.


a
% Active flies
Time, min
Axn[EY10228]/+
TM3/+
Axn[EY10228]/TM3
Ctl
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84
0 20 40 60 80 100 120


b
% Recovered flies
Time, min
Axn[EY10228]/+
TM3/+
Axn[EY10228]/TM3
Ctl
0 3 6 9 12 15 18 21 24 27 30 33 36
0 20 40 60 80 100 120


c
MST, min
0 10 20 30 40 50 60 70 80
Ctl
Axn[EY10228]/+
TM3/+
Axn[EY10228]/TM3
*
*
*


d
MRT, min
0 5 10 15 20 25 30
Ctl
Axn[EY10228]/+
TM3/+
Axn[EY10228]/TM3
*
*
*

4.5.3 *Hiw* and Ethanol Sensitivity

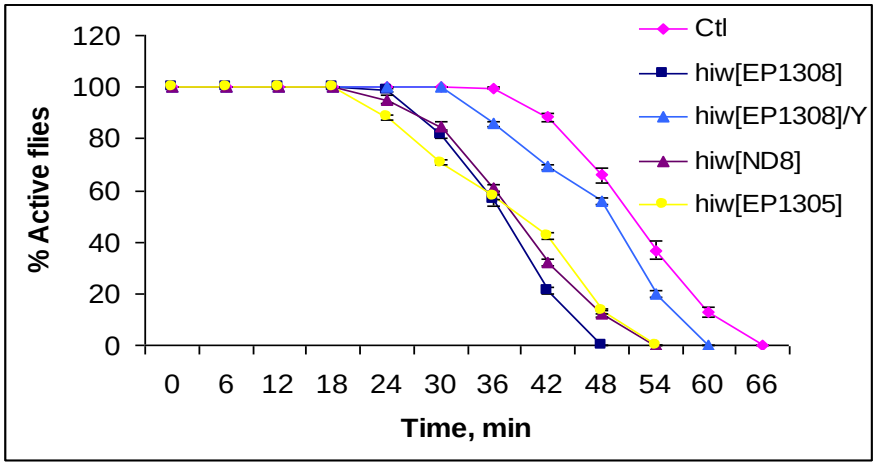
Drosophila highwire encodes a ubiquitin ligase protein involved in the negative regulation of synaptic growth at the *Drosophila* NMJ. All *hiw* alleles are viable. The hiw^{EP1308} allele is a *P* element insertion mapping to the 5' untranslated region of the first exon (Wan et al., 2000). It was observed that flies homozygous for the hiw^{EP1308} showed increase in sensitivity in response to ethanol sedation and recover significantly slower when compared to the control flies (Table 4.1, Figure 4.7) suggesting that a functional *hiw* gene is required for the normal sensitivity to ethanol.

To test whether hiw^{EP1308} causes ethanol sensitivity phenotype in a dominant negative manner, homozygous $hiw^{EP1308}/hiw^{EP1308}$ female flies were crossed to wild-type strain 2447 males to generate hemizygous hiw^{EP1308}/Y flies. The behaviour of these flies was not significantly different from wild-type in the sedation assay and they recovered normally from the sedative effect of ethanol in the recovery assay (Figure 4.7), indicating that the mutation is not due to dominant phenotype associated with hiw^{EP1308}. This also indicates that a single functional copy of hiw gene is sufficient for normal sensitivity to the sedative effect of ethanol in both assays. However, it is possible that it is not the *P* element insertion in the *hiw* gene that is responsible for this phenotype and that hiw^{EP1308} carries a second unidentified mutation or allelic variant(s) that leads to the ethanol sensitivity phenotype. To test this hypothesis, other independently isolated alleles of hiw were tested in the 2 behavioural paradigms. Two strains hiw^{EP1305} and hiw^{ND8} were tested for ethanol sensitivity. Hiw^{EP1305} is caused by *P* element insertion at the 3' end of the large intron while the hiw^{ND8} is a nonsense allele expressing truncated protein (Wu et al., 2005) and has been shown to behave like a loss-of-function allele (Wu et al., 2005). Both alleles showed increase sensitivity in response to the sedative effect of ethanol and their rate of recovery from ethanol sedation were significantly less than that of the control flies (Figure 4.7). A similar difference to wild type was observed when female *hiw* flies for all these mutants were tested in sedation assay and they also recovered significantly slower than the wild-type control. Taken together, this confirms that *hiw* function is required for normal ethanol sensitivity in *Drosophila*.

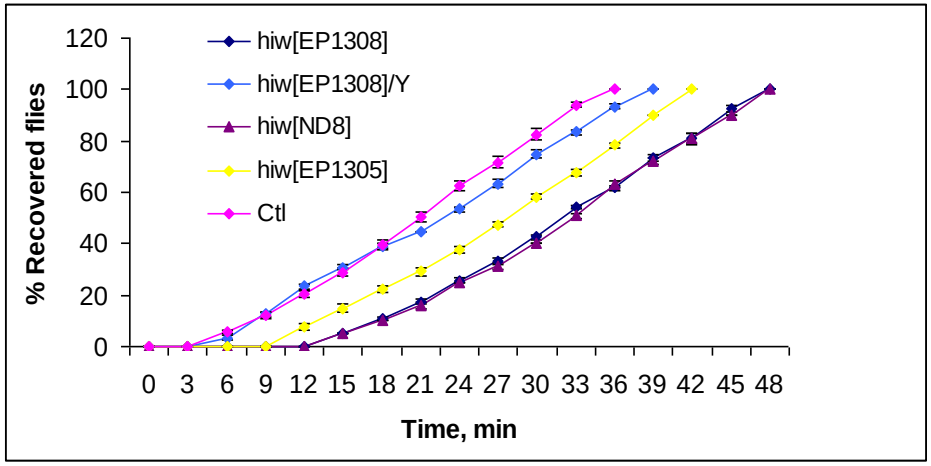
Figure 4.7 | *hiw* flies have increased ethanol sensitivity.

(a) Left panel shows the mean sedation profiles for flies exposed to ethanol. **(b)** Shows the recovery time from ethanol intoxication of these flies. **(a** and **b)** *hiw* mutants (*hiw*^{EP1305}, *hiw*^{EP1308} and *hiw*^{ND8}) show enhanced ethanol sensitivity compared with wild-type control, Ctl. **(c** and **d)** Mean sedation time (MST) and mean recovery time measuring the flies' resistance to and recovering from the sedative effects of ethanol respectively. *hiw* mutants show significant increased ethanol sensitivity compared with wild-type control, (Ctl). $^{*}P < 0.0001$**;** n=6-20 experiments. In all panels errors bars represent SEM.

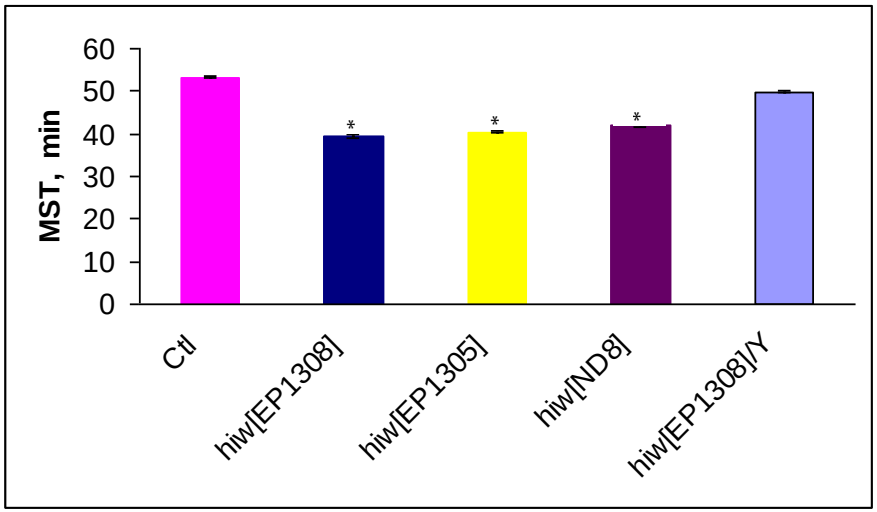
a


% Active flies
120
100
80
60
40
20
0
0 6 12 18 24 30 36 42 48 54 60 66
Time, min
Ctl
hiw[EP1308]
hiw[EP1308]/Y
hiw[ND8]
hiw[EP1305]

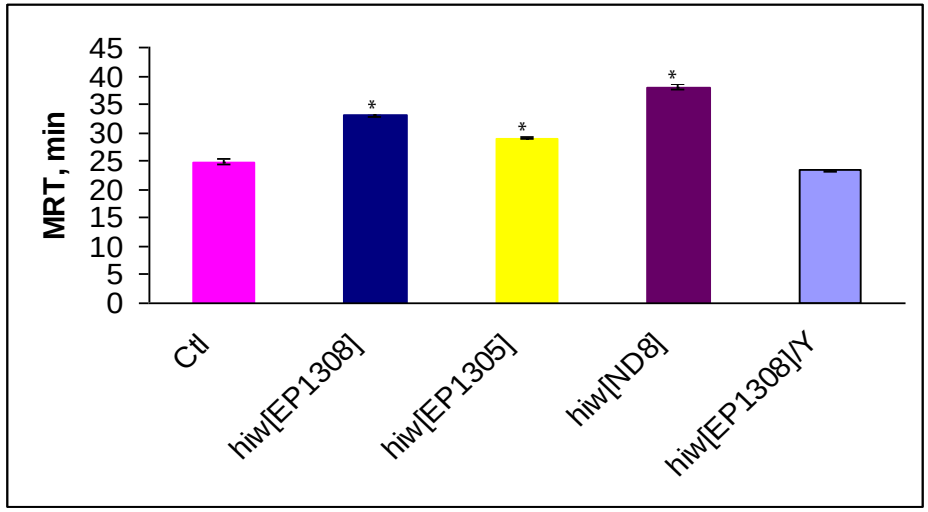
b


% Recovered flies
120
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
Time, min
hiw[EP1308]
hiw[EP1308]/Y
hiw[ND8]
hiw[EP1305]
Ctl

c


MST, min
60
50
40
30
20
10
0
*
*
*
Ctl
hiw[EP1308]
hiw[EP1305]
hiw[ND8]
hiw[EP1308]/Y

d


MRT, min
45
40
35
30
25
20
15
10
5
0
*
*
*
Ctl
hiw[EP1308]
hiw[EP1305]
hiw[ND8]
hiw[EP1308]/Y

4.5.4 *hop* and Ethanol Sensitivity

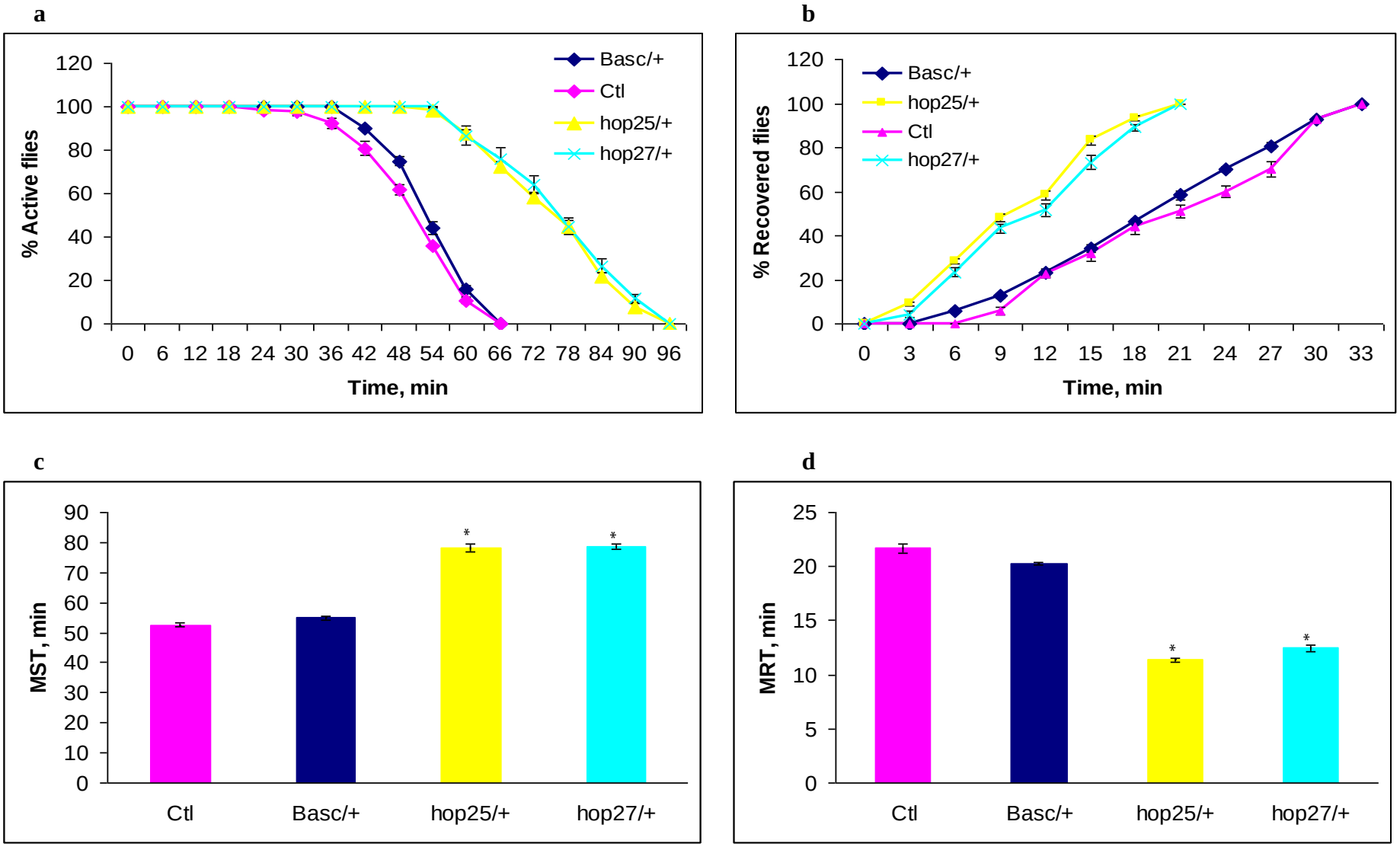
The molecular lesions of the two *hop* mutants tested in both sedation and recovery assays reside in separate domains of the predicted protein (Luo et al., 1999). Molecular characterisation of the *hop* mutations have shown that hop^{27} (also known as hop^{M4}) is located in the kinase domain while hop^{25} (also known as hop^{MSV1}) is located in JH6 domain as determined from the HOP nucleic acid sequence for these two mutations (Luo et al., 1999). The mutations in these two *hop* mutants are chemically induced resulting in point mutations (hop^{25} is induced by EMS while hop^{27} by ENS). These two mutants are recessive lethal and, as *hop* is on the X chromosome, males are not viable and so heterozygous female flies were used.

The hop^{27} allele is a loss of function mutation. It was observed that flies heterozygous for the hop^{27} mutation displayed a statistically significant decreased in ethanol sensitivity compared with either *Basc*/+ (obtained from a cross between virgin hop^{27} /*Basc* and male wild-type 2447 (Ctl)) or wild-type control ($P<0.0001$; Figure 4.8). Similarly, these flies recovered significantly faster than either of *Basc*/+ or wild-type control ($P< 0.0001$, Figure 4.8). The hypomorphic mutation, hop^{25} also exhibited this reduced sensitivity to ethanol sedation ($P<0.0001$; Figure 4.8).

The ethanol phenotype observed in *hop* mutant flies is specific. Mutations in the two *hop* alleles tested are located in different domains/ regions of the HOP protein and are caused by different mutagenic chemicals. The display of similar ethanol behavioural responses by the *hop* alleles therefore strongly indicates the specificity of the ethanol phenotypes of the *hop* mutants.

Figure 4.8 | *hop* flies have reduced ethanol sensitivity.

(a) Left panel shows the mean sedation profiles for flies exposed to ethanol. **(b)** Shows the recovery time from ethanol intoxication of these flies. **(a** and **b)** *hop* mutants (hop^{25}/+ and hop^{27}/+) show reduced ethanol sensitivity compared with two controls, Ctl and *Basc*/+. **(c and d)** Mean sedation time (MST) and mean recovery time measuring the flies' resistance to and recovering from the sedative effects of ethanol respectively. *hop* mutants show significantly reduced ethanol sensitivity compared with two controls, Ctl and *Basc*/+. $^{*}P < 0.0001$**;** n=6-20 experiments. In all panels errors bars represent SEM.


a
% Active flies
Time, min
Basc/+
Ctl
hop25/+
hop27/+
b
% Recovered flies
Time, min
Basc/+
hop25/+
Ctl
hop27/+
c
MST, min
Ctl
Basc/+
hop25/+
hop27/+
d
MRT, min
Ctl
Basc/+
hop25/+
hop27/+

4.5.5 *Hsp26* and Ethanol Sensitivity

After an exposure to an acute dose of ethanol, the microarray data show that *hsp26* is the most highly upregulated gene tested. To determine whether this gene regulates ethanol sensitivity, two viable *hsp26* mutants were tested in the sedation and recovery assays. These two *hsp26* mutants are caused by different P element insertions at exactly the same position near the 5' end of the untranslated exon and they correspond to different P elements (EPgy2 in $hsp26^{EY10556}$ and SUPor-P in $hsp26^{KG027861}$).

When $hsp26^{EY10556}$ is tested in sedation and recovery assays, two different interesting observations were noted: male flies homozygous for $hsp26^{EY10556}$ did not exhibit a significant difference to the sedative effects of ethanol in sedation assay compared with wild-type control ($P = 0.48$; Figure 4.9) but recovered marginally significantly faster with a lower MRT compared with wild-type control ($P < 0.01$; Figure 4.9).

It is postulated that the P element insertion in the gene is causing the phenotypes and not effect due to genetic background. If this is correct, the second mutant $hsp26^{KG027861}$ with different P element in the same insertion point should result in similar behavioural phenotypes. As expected, when this mutant was tested in both paradigms, similar behaviours were seen (Figure 4.9).

However, unlike the male flies, female $hsp26^{EY10556}$ were less sensitive to ethanol in the sedation assay having a MST of 62.9±0.4 compared with either of male $hsp26^{EY10556}$ (MST 54±0.2) and wild-type female controls (MST 52.6±0.7) [$P < 0.0001$; Figure 4.9] indicating sex specific effects of ethanol-induced sensitivity for sedation assay in *hsp26* deficient *Drosophila*. These intriguing results suggest that involved mechanisms might contribute differently in the two sexes. The dissimilar ethanol sensitivity effects for sedation assay in males and females probably may be partly explained by their differences in response to Hsp deficiency (Sørensen et al., 2007). Different responses to Hsp deficiency have been suggested to contribute in part to sex-specific differences in heat-induced hormesis in Hsf-deficient *Drosophila.* It will therefore be interesting to determine separately the expression level of *hsp26*

male and female flies after acute ethanol exposure and reason whether this might help to shed light on the significant effect of sex in *hsp26* flies.

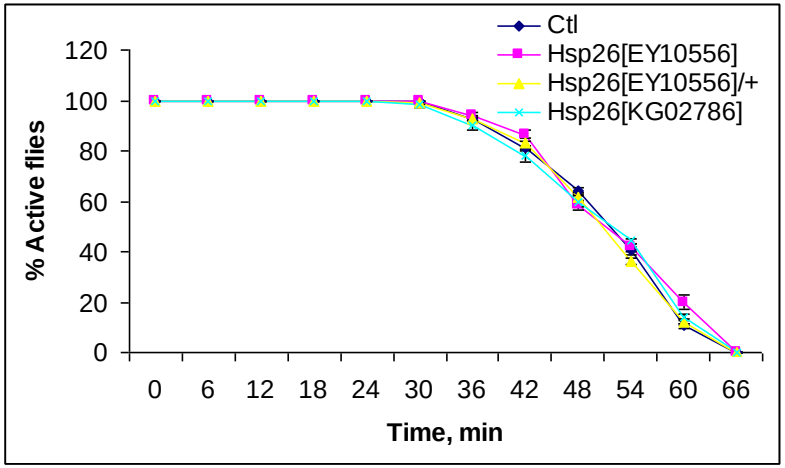
Finally, given that most of the basic regulatory mechanism governing the behaviour of eukaryotic cells are conserved between *Drosophila* and mammals (Cowmeadow et al., 2005) and the fact that heat shock pathways are conserved in evolution (Scholz et al., 2005) indicate the conservation of this *hsp26* pathway.

Figure 4.9 | *hsp26* flies have normal ethanol sensitivity only in the sedation assay.

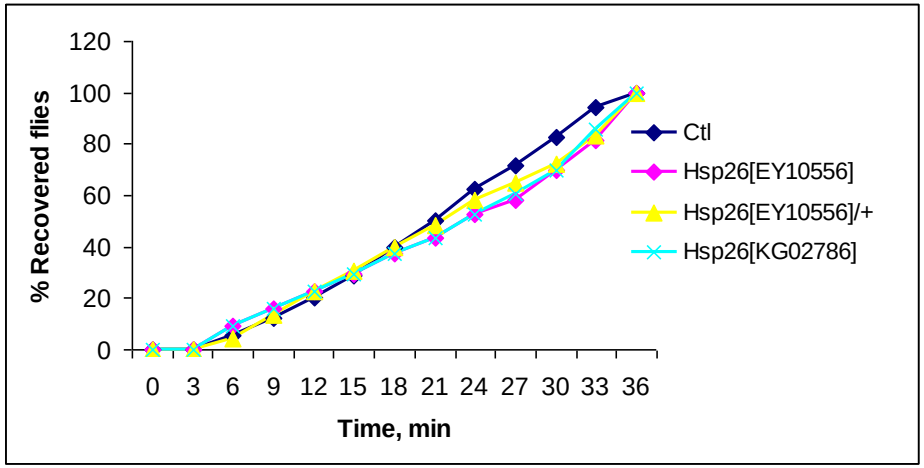
(a) Left panel shows the mean sedation profiles for flies exposed to ethanol. **(b)** Shows the recovery time from ethanol intoxication of these flies. **(a** and **b)** *hsp26* mutants (*hsp26*^{EY10556} and *hsp26*^{KG027861}) show normal ethanol sensitivity behaviour compared with wild-type (Ctl) flies.

(c and **d)** Mean sedation time (MST) and mean recovery time (MRT) measuring the flies' resistance to and recovering from the sedative effects of ethanol respectively. *hsp26*^{EY10556} and *hsp26*^{KG027861} flies showed normal MST **(c)** but significantly reduced MRT ($^{*}P < 0.05$) **(d)** compared with control**;** n=6-20 experiments. In all panels errors bars represent SEM.

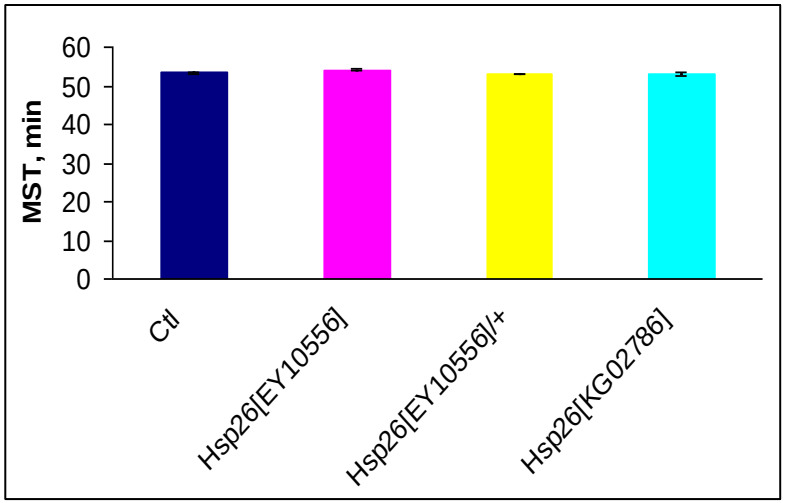
a


Ctl
Hsp26[EY10556]
Hsp26[EY10556]/+
Hsp26[KG02786]
% Active flies
120
100
80
60
40
20
0
0 6 12 18 24 30 36 42 48 54 60 66
Time, min

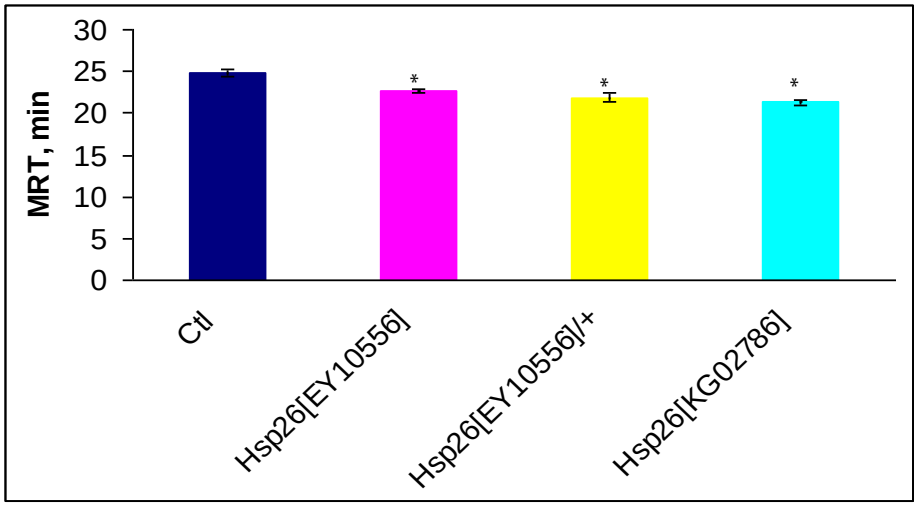
b


% Recovered flies
120
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30 33 36
Time, min
Ctl
Hsp26[EY10556]
Hsp26[EY10556]/+
Hsp26[KG02786]

c


MST, min
60
50
40
30
20
10
0
Ctl
Hsp26[EY10556]
Hsp26[EY10556]/+
Hsp26[KG02786]

d


MRT, min
30
25
20
15
10
5
0
*
*
*
Ctl
Hsp26[EY10556]
Hsp26[EY10556]/+
Hsp26[KG02786]

4.5.6 *Hsp83* and Ethanol Sensitivity

Hsp83^{08445} (known as *scratch*) was obtained in a *P* element insertional mutation screen (Karpen and Spradling, 1992; Castrillon et al., 1993). The *P* element in *scratch* is inserted in the intron of the *hsp83* gene located approximately 60bp from the junction of the first exon and the intron (Yue et al. 1999). This mutant is homozygous viable, female fertile but male sterile (Castrillon et al., 1993) and it is maintained over a third chromosome balancer (*TM3*). It was observed that flies homozygous for *scratch* showed reduced sensitivity to ethanol and recovered significantly faster when compared with either wild-type control or *hsp83*^{08445}/*TM3* flies (Figure 4.10), suggesting that a functional *hsp83* gene is required for the normal sensitivity to ethanol. Interestingly and surprisingly, there are no statistically significant differences between the wild-type control and *hsp83*^{08445}/*TM3* flies ethanol response in both the sedation and recovery assays (Figure 4.10). One possible explanation for this is that *hsp83*^{08445}/*TM3* ethanol phenotype represents the balanced sum of sensitivity effects caused by both *hsp83*^{08445} and *TM3* in the heterozygous *hsp83*^{08445}/*TM3*. It is also possible that *TM3* compensates for the ethanol sensitivity of *hsp83*^{08445} at a relatively normal level and that *TM3* is epistatic to the ethanol resistance causing *hsp83*^{08445} allele. Whatever may be the actual mechanism involved, the behaviour seen in *hsp83*^{08445}/*TM3* flies clearly indicate that direct comparisons between the homozygous *hsp83*^{08445}/ *hsp83*^{08445} and the wild-type control flies could be made.

Similarly, another mutant allele *hsp83*^{e6A} (recessive lethal and maintained over a balancer chromosome *TM6B*), displayed a reduced ethanol sensitivity that was statistically different from either the *TM6B*/+ (obtained from a cross between *hsp83*^{e6A}/*TM6B* and wild-type flies, Table 4.1) or the wild-type controls ($P<0.0001$; Figure 4.10). This mutation is caused by an EMS-induced mutation (point mutation) in the coding region of *hsp83* in the position of the amino acid exchanges S592F (C1775T) within the C-terminal protein domain (Yue et al., 1999). The result indicates that reduced sensitivity to ethanol is present even in heterozygous flies with reduced *hsp83* gene product.

It should however, be noted that both alleles of *hsp83* displayed a reduced ethanol sensitivity that is statistically significantly different from each other ($P < 0.001$, Figure 4.10) indicating functional *hsp83* dose level responses to ethanol sensitivity in *Drosophila*. This marked difference between the two *hsp83* mutants is most likely due to their different levels of activity in the *hsp83* genome; while the EMS mutation in $hsp83^{e6A}$ flies is an hypomorphic mutation, the *P* element in *scratch* is in the intron, resulting in a small reduction in the wild-type Hsp90 function (Yu et al.,1999).

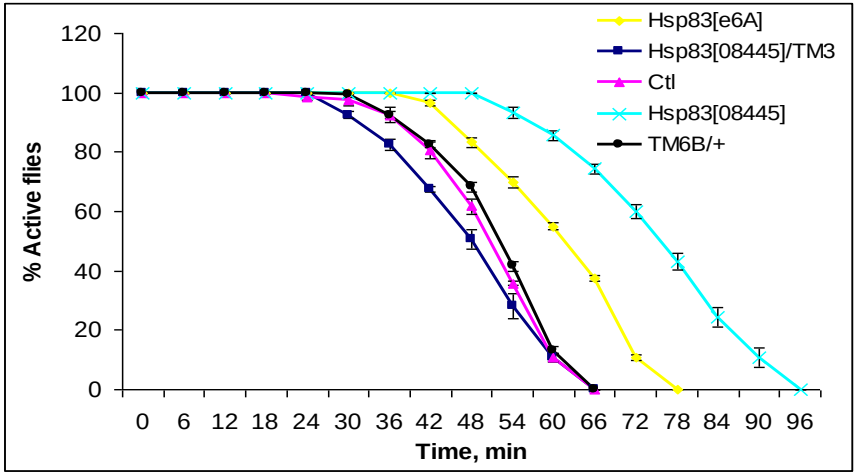
Finally, though the *hsp83* mutants are highly pleiotropic (Yue et al., 1999), the ethanol sensitivity defects seen in both alleles in different genetic background strongly suggests their relative specific role in ethanol regulation in *Drosophila*.

Figure 4.10 | *hsp83* flies have reduced ethanol sensitivity.

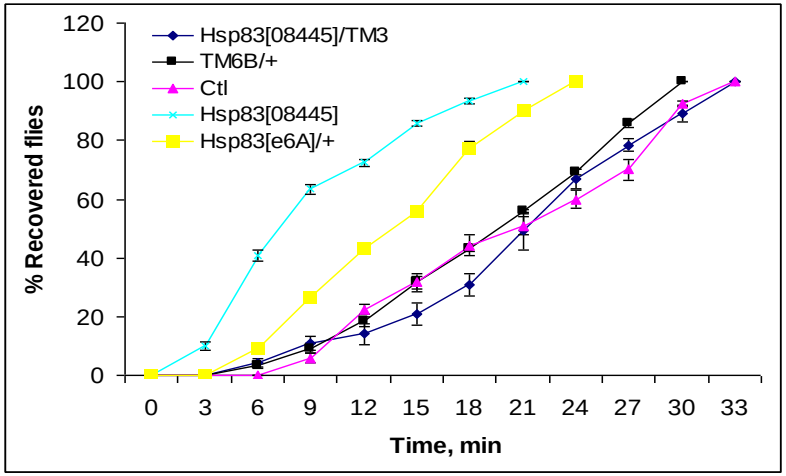
(a) Left panel shows the mean sedation profiles for flies exposed to ethanol. **(b)** Shows the recovery time from ethanol intoxication of these flies. **(a** and **b)** *hsp83* mutants (*hsp83*^{08445} and *hsp83*^{e6A}) show reduced ethanol sensitivity compared with Ctl, *hsp83*^{08445}/*TM3*, and *TM6B*/+. **(c** and **d)** Mean sedation time (MST) and mean recovery time measuring the flies' resistance to and recovering from the sedative effects of ethanol respectively. *hsp83* mutants show significantly reduced ethanol sensitivity compared with controls.

$^{*}P < 0.0001$; n=6-20 experiments. In all panels errors bars represent SEM.

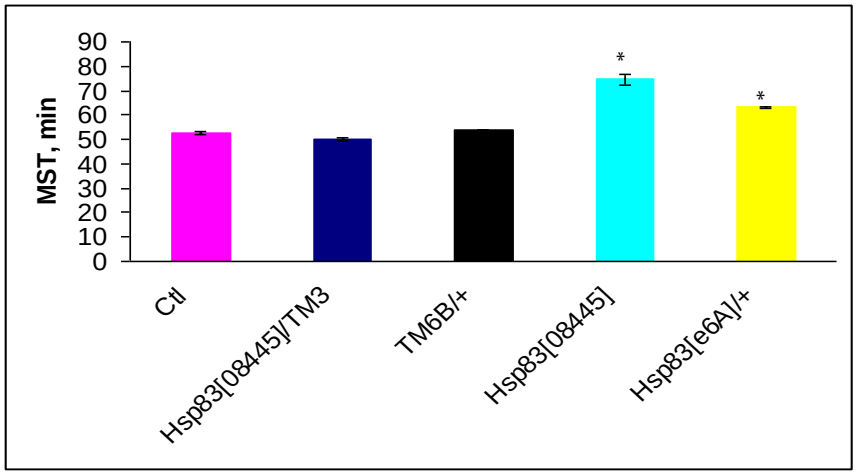
a


Hsp83[e6A]
Hsp83[08445]/TM3
Ctl
Hsp83[08445]
TM6B/+
% Active flies
Time, min
0
20
40
60
80
100
120
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96

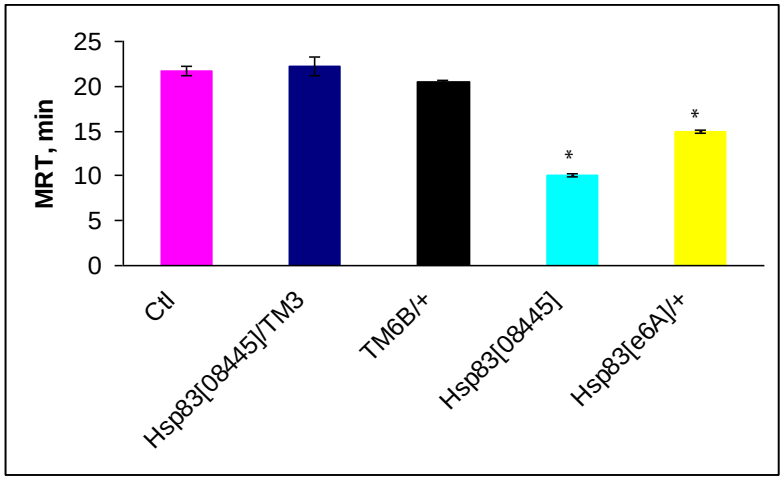
b


Hsp83[08445]/TM3
TM6B/+
Ctl
Hsp83[08445]
Hsp83[e6A]/+
% Recovered flies
Time, min
0
20
40
60
80
100
120
0 3 6 9 12 15 18 21 24 27 30 33

c


MST, min
0
10
20
30
40
50
60
70
80
90
*
*
Ctl
Hsp83[08445]/TM3
TM6B/+
Hsp83[08445]
Hsp83[e6A]/+

d


MRT, min
0
5
10
15
20
25
*
*
Ctl
Hsp83[08445]/TM3
TM6B/+
Hsp83[08445]
Hsp83[e6A]/+

4.5.7 *mbf1* and Ethanol Sensitivity

Drosophila multiprotein bridging factor 1 encodes a stress response protein that functions as a transcriptional co-activator. The *P* element in $mbf1^2$ flies tested in sedation and recovery assays is inserted 21bp upstream of the first exon of the mbf1 gene. This $mbf1^2$ is a null allele, as the ~1.6kb transcript encoded by its protein are undetectable in the mutant as confirmed by southern blot, and western blot analyses of the *mbf1* protein from adult flies (Jindra et al., 2004). A rescue construct that includes a genomic *mbf1* in an $mbf1^2$ mutant background, P[$mbf1^+$]; $mbf1^2$, was used to control for effects due to genetic background.

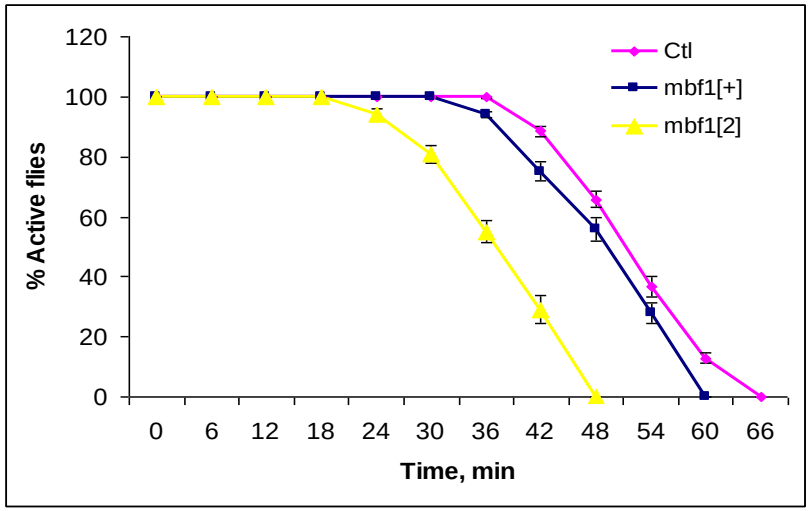
To test the possibility that the loss of $mbf1^2$ affects ethanol sensitivity in *Drosophila*, the comparison between the ethanol resistance of the $mbf1^2$ mutants and wild-type flies was first made in the presence of 50% ethanol vapour for sedation assay. It was observed that $mbf1^2$ animals showed enhanced sensitivity to ethanol compared with wild-type control strain ($P < 0.0001$; Figure 4.11). Similarly to test the rate of recovery from the sedative effect of ethanol, male $mbf1^2$ flies were assayed in the recovery paradigm. The mean recovery time (MRT) of the $mbf1^2$ homozygous was 40.8 ± 0.9 compared to 24.9 ± 0.4 of the wild-type strain.

To demonstrate that the differential ethanol sensitivity seen between $mbf1^2$ and wild-type control flies is not due to genetic background, sedation and recovery assays were performed on the line P[$mbf1^+$]; $mbf1^2$ (designated $mbf1^+$) that contains an insertion of the $mbf1^2$ gene in an $mbf1^2$ background (Liu et al., 2003). $mbf1^+$ flies displayed normal sedation and recovery assay behaviour (Figure 4.11). Given that $mbf1^+$ and $mbf1^2$ flies are in the same genetic background, it is therefore, very unlikely that the sensitivity observed was caused by another mutation in the $mbf1^-$ or $mbf1^+$ chromosomes. Together, these results show that the loss of *mbf1* renders animal sensitive to ethanol.

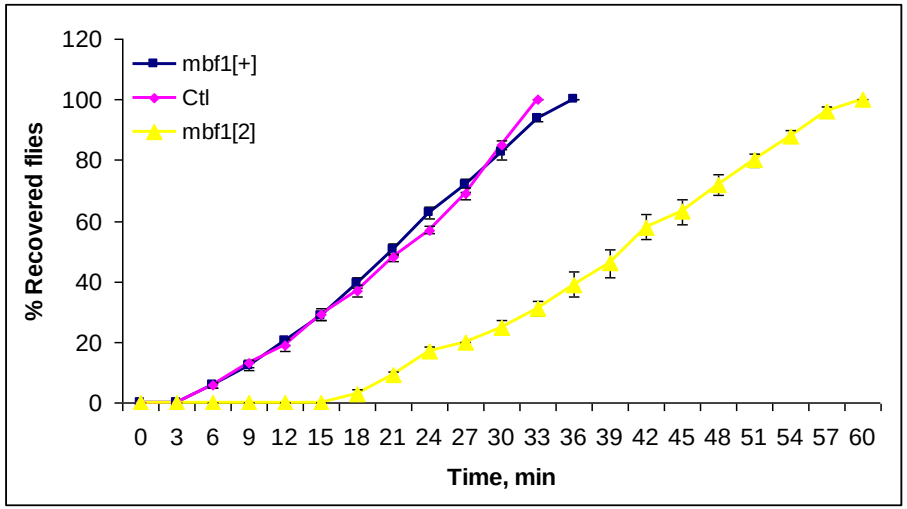
Figure 4.11 | *mbf1*^{2} flies have increased ethanol sensitivity.

(a) Left panel shows the mean sedation profiles for flies exposed to ethanol. **(b)** Shows the recovery time from ethanol intoxication of these flies. In **(a** and **b)** mbf1^{2} flies show enhanced ethanol sensitivity compared with two controls, (Ctl and *mbf1*$^{+}$). **(c** and **d)** Mean sedation time (MST) and mean recovery time quantifying the flies' resistance to and recovering from the sedative effects of ethanol respectively. *mbf1*^{2} mutants show significantly increased ethanol sensitivity compared with two controls, (Ctl and *mbf1*$^{+}$). $^{*}P < 0.0001$**;** n=6-20 experiments. In all panels errors bars represent SEM.

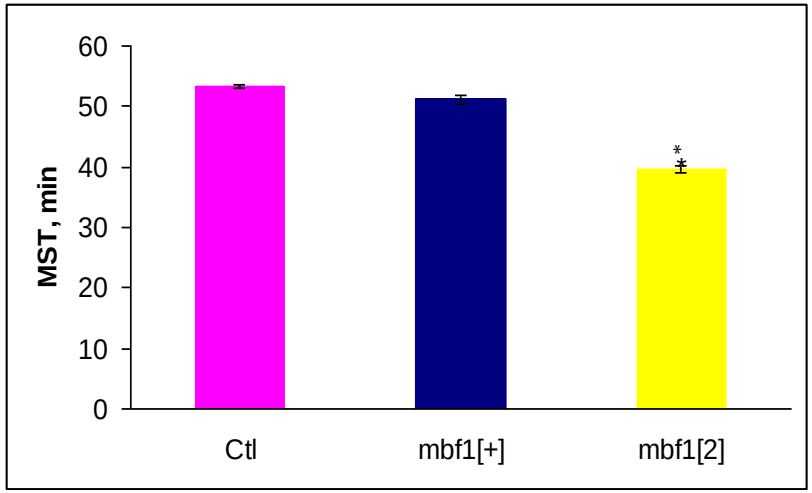
a


120
100
80
60
40
20
0
% Active flies
0 6 12 18 24 30 36 42 48 54 60 66
Time, min
Ctl
mbf1[+]
mbf1[2]

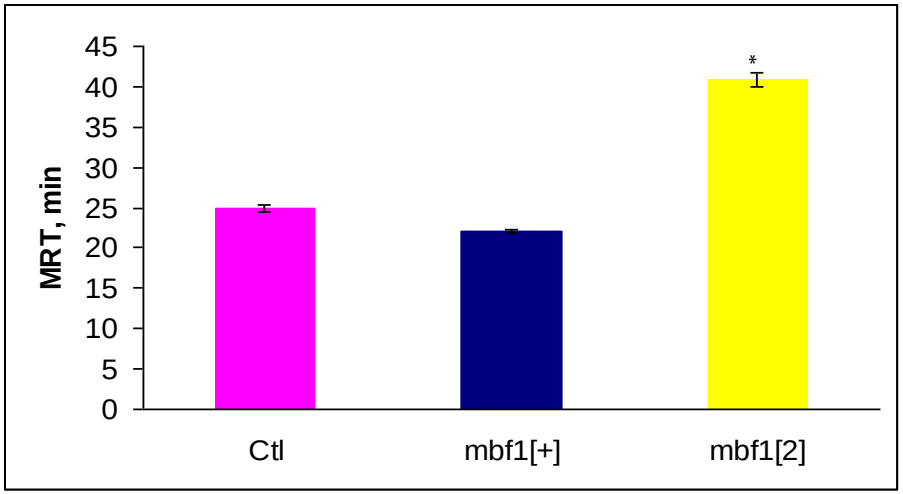
b


120
100
80
60
40
20
0
% Recovered flies
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60
Time, min
mbf1[+]
Ctl
mbf1[2]

c


60
50
40
30
20
10
0
MST, min
*
Ctl
mbf1[+]
mbf1[2]

d


45
40
35
30
25
20
15
10
5
0
MRT, min
*
Ctl
mbf1[+]
mbf1[2]

4.6 Chapter Discussion

Behavioural geneticists have used a number of paradigms to screen for novel genes implicated in ethanol response. However, it should be noted that such response to ethanol is distributed and so different paradigms measure different aspects of ethanol response. Thus, direct comparison between assays that quantify different aspects of ethanol response should be treated with caution. Given that ethanol response is distributed, studies have highlighted the importance of assessing the effects of genetic differences or dissociation in multiple behavioural tests (Crabbe et al., 2002; Berger et al., 2004). In this study, sedation and recovery assays measuring the fly's resistance to the sedative effect of ethanol were employed (Wen et al., 2005). These two assays involved the use of different methodologies in quantifying different aspects of ethanol sensitivity: while sedation assay measures fly's resistance to the sedative effect of ethanol, recovery assay on the other hand, measures the ability of flies to recover from ethanol's sedative effect.

Of the 7 mutant genes examined, 6 of them exhibited alterations in ethanol sensitivity in the sedation assay and all of them in the recovery assay. The implication of all these genes in ethanol sensitivity is not surprising and in fact validates the very high stringency used in selecting them for testing. It is thus, very interesting to ask whether the mechanisms that regulate the sedation of flies in the sedation assay are distinct from those that regulate the recovery of flies in the recovery assay. Among the ethanol-sensitivity mutants, 10 of the 12 tested showed a defect in both behavioural assays (Figure 4.4), suggesting, that for the most part, the effects of mutations on fly's resistance to and recovery from ethanol sedative effects are similar. Two *P* elements insertion corresponding to the same gene ($hsp26^{EY10556}$ and $hsp26^{KG027861}$), showed a normal behaviour in the sedation assay yet displayed quicker recovery from ethanol's sedative effects in the recovery assay compared with wild-type control, indicating that genetic dissociation really exists between assays that quantify distinct aspect of ethanol sedation.

In this study, while 4 mutant genes exhibited decreased sensitivity to ethanol in sedation assay, only 2 exhibited increased sensitivity (Table 4.1, Figure 4.4). Similarly, in the recovery assay, mutants exhibiting reduced sensitivity were much

more frequent (5 mutant genes) than those with enhanced sensitivity (2 mutant genes). In a related manner, a recent analysis of ethanol sensitivity of 20 genes identified from microarray analysis using the inebriometer, a paradigm that measures the fly's postural control, identified 7 mutant genes exhibiting decreased sensitivity and only 1 with increased sensitivity (Morozova et al., 2006; Berger et al., 2008). Although these studies were carried out in a different behavioural paradigm than ours (inebriometer vs. sedation/recovery assays), very similar results were obtained, suggesting similar trends in the phenomena of increase and decrease sensitivity to ethanol involved in both studies.

The identification of genes potentially implicated in ethanol sensitivity suggests underlying pathways /mechanisms in acute ethanol action. For example, a role for the appropriate regulation of ubiquitination pathways in ethanol sensitivity is further suggested by the genes implicated in one of the increased sensitivity mutants [*hiw*, encoding a RING finger domain required for protein ubiquitination by acting as an ubiquitin ligase (Wu et al., 2005)]. A detailed discussion on *hiw*-mediated ubiquitination pathway in relation to ethanol sensitivity is given in sections 5.6 and 6.4 of Chapters 5 and 6 respectively.

Four genes suggesting a functional implication of kinase signaling pathways in ethanol sensitivity have been discovered: [*hsp83,* required for a raf-mediated signaling pathway (van der Straten et al., 1997), encodes a highly conserved Hsp90 protein reported to be involved in a variety of processes including signal transduction and protein trafficking (Yue et al., 1999) (see section 5.6 of Chapter 5 for a detailed discussion on the role of Hsp90 in ethanol response) *hiw*, encoding a large neuronal protein required for presynaptic BMP signaling essential for synaptic growth (McCabe et al., 2004), *hop*, encoding a non-receptor tyrosine kinase which is involved in the JAK/STAT signaling pathway (Binari and Perrimon 1994; Luo et al., 1999); and *Axn*, shown to negatively regulate wg signaling pathway (Willert et al., 1999)]. Indeed, a link between the JAK/STAT signaling pathway and the involvement of Ras/Raf/MAPK and wingless signaling pathway in *Drosophila* has been suggested (Aggaise and Perrimon, 2004; Ekas et al., 2006). For instance, evidence indicates that *Drosophila hop* physically interacts with *Drosophila*-Raf (D-

Raf) in vitro and D-Raf is a direct transcriptional target for STAT activation with the Raf promoter containing consensus STAT-binding sites (Aggaise and Perrimon, 2004). Thus, the broadly defined signaling pathway encoded by these upregulated genes in ethanol response strongly suggests a coordinated defense mechanism aimed at protecting the cells from toxic effects of ethanol. These genes may also mediate longer term changes to ethanol exposure.

Other genes displaying altered ethanol sensitivity hint at roles for neurogenesis [*ana*, encoding a glycoprotein secreted by glial cells and involved in negative regulation of neuroblast proliferation (Ebens et al., 1993); and oxidative stress response (*mbf1*, involved in reduced life span (Jindra et al., 2004); *hsp26* shown to extend life span in *Drosophila* (Wang et al. 2004, Liao et al., 2008); and circadian regulation (*hsp83*, involved in homeostatic response to sleep deprivation in *Drosophila* (Shaw et al. 2002)]. *ana* mutant third instar larvae have been shown to display reduced olfactory sensitivity to several odorants including ethyl acetate compared to controls while no significant olfactory phenotype was seen in the trials of *ana* mutants adults with ethyl acetate at several concentrations in a T-maze behavioural paradigm (Park et al. 1997). Thus, the role of adult *ana* mutant flies in decreased ethanol sensitivity in the current study may suggest a specific behavioural response to ethanol by the *ana* gene. *Drosophila mbf1* role has been reported to be critical when gene expression is required in response to developmental or environmental signals (Liu et al., 2003). This gene is also suggested to be involved in Ca^{2+}-induced gene activation (Liu et al., 2003) with the role of calcium in the acute action of, and development of tolerance to, ethanol has long been documented (Mayer et al., 1980).

More importantly, the observation that the upregulation of certain chaperone proteins, in this case *hsp26* and *hsp83*, affects the fly's sensitivity to ethanol provides a useful hint about the functional targets of ethanol and its molecular mechanisms. The fact that these two chaperone genes behave differently in sedation assay further suggest different mechanistic processes underpinning their behavioural responses to the sedative effects of ethanol.

Finally, having established that all the mutants tested showed sensitivity to ethanol in at least one of the two assays, it will be interesting to test some of these genes for ethanol tolerance. This is necessary in order to define genes functionally involved in ethanol response and could later be manipulated in different brain regions for ethanol sensitivity and tolerance. Tolerance in this way is expected to shed more light on these genes in terms of the pathways and/or mechanisms underlying their behavioural responses to ethanol. Thus, from the ethanol sensitivity data collected, all the genes look very promising and are worth investigating further. However, for further work, the most interesting and viable studies are suggested by the results from the chaperone mutants (*hsp26*^{EY10556}, *hsp26*^{KG027861} and *hsp83*^{08445} and *hsp83*^{08445}), *mbf1*^{2} and *hiw* populations. Accordingly, further work was completed on these four promising and interesting genes in Chapter 5.

Chapter 5.

Testing Selected Genes for Rapid Ethanol Tolerance.

5 Testing Selected Genes for Rapid Ethanol Tolerance

The work described in this chapter describes experiments to test four of the genes validated in Chapter 4 for ethanol tolerance. Again the approach taken is to use flies carrying mutations in these genes in a series of tolerance test. Section 5.1 provides a brief introduction to the study and describes the need for tolerance testing on these selected genes. Section 5.2 explains the experimental procedure and sections 5.3 to 5.5 present analyses of the results. Finally, section 5.6 summarises the conclusion of the investigation.

5.1 Introduction

Alcohol consumption in man causes long-term physiological changes including tolerance. Tolerance in this sense can develop to both the pleasurable (e.g. euphoria and loss of social inhibition) and the aversive (e.g. loss of motor coordination and sedation) effects of ethanol (Fadda and Rossetti, 1998), and thought to encourage increased alcohol intake, development of physical dependence and addiction (Tabakoff et al., 1986). Indeed, alcoholics acquire extra-ordinary tolerance to the intoxicating effects of ethanol, usually associated with dependence and uncontrolled craving to continue drinking (reviewed in Diamond and Gordon, 1997).

Tolerance is defined as a reduction in drug responsiveness seen after a repeated exposure to that drug. There are believed to be different types of tolerance: Acute tolerance, which occurs within drug session/ experience. Rapid tolerance, which occurs after the completion of a single drug exposure/ experience, and chronic tolerance arises from serial drug exposures (Berger et al., 2004). Further, two mechanisms of tolerance which are not mutually exclusive have been reported: metabolic/pharmacokinetic tolerance, involving changes in the disposition of ethanol (such as absorption, excretion or metabolism) leading to efficient removal of alcohol from the body and functional/pharmacodynamic tolerance involving changes experienced at a cellular level and mediated by adaptations in neural function (reviewed in Faida and Rosetti, 1998).

Drosophila has been shown to acquire rapid tolerance to the sedating effects of ethanol (Scholz et al., 2000). Thus, in flies many genes have been implicated in ethanol tolerance. For example, flies carrying a mutation in the *hangover (hang)* gene (a gene encoding a nucleic acid binding zinc finger protein) were implicated in reduced ethanol tolerance development in the same manner as flies lacking the neuromodulator octopamine owing to a mutation in the gene encoding tyramine B hydroxylase (Tbh) (Scholz et al., 2000; 2005). Further, induction of tolerance was completely abolished in flies carrying both null mutations in *hang* and in the gene encoding *Tbh*, indicating that both genes are involved in different pathways in the induction of ethanol tolerance (Scholz et al., 2005). Flies carrying *slowpoke*, a Ca^{2+}-activated K^+ channel gene which is a critical modulator of neuronal excitability has also been shown to be required for the acquisition of tolerance to ethanol (Cowmeadow et al., 2005; Cowmeadow et al., 2006). The *Drosophila* homologue of the *jwa* gene encoding a large PRA1 domain was also reported to be necessary for the acquisition of ethanol tolerance in *Drosophila* (Li et al., 2008). Finally, a study of long-term memory mutants flies implicated several genes in reduced ethanol tolerance including *exba* (*elF-5C*), a translational regulator and involved in axon guidance); *pumilio* (translational regulator) and *formin3*, involved in actin assembly (Berger et al., 2008)

The microarray experiments have been used to profile changes in gene expression following acute ethanol exposure in *Drosophila*. Using microarrays, many genes exhibiting adaptive changes in gene expression in response to an acute dose of ethanol treatments were identified. Seven of these genes were validated and linked to ethanol sensitivities (mutant analyses) in flies in Chapter 4. Of these seven genes, four genes namely; *hsp26*, *hsp83*, *mbf1* and *hiw* were selected for rapid ethanol tolerance studies in this Chapter. To determine whether these selected genes mediate some of the behavioural adaptations underlying ethanol tolerance in *Drosophila*, individual flies carrying mutations in these genes were tested for rapid tolerance. Rapid tolerance in this sense is defined as the attenuated response to ethanol that occurs from a single exposure and after an elevated blood level from the initial exposure have returned to near basal levels (Crabbe et al., 1979).

5.2 Methods

The assay to measure ethanol tolerance of these genes was based on the sedation protocol (see section 2.3.4 of the Materials and Methods). To quantify rapid tolerance, flies were first tested in the sedation assay as in the case of normal ethanol sensitivities, given sufficient time to recover from this first sedation and metabolise the ethanol, and then re-tested in the sedation paradigm.

5.3 Rapid tolerance can be measured with the sedation assay.

It has previously been reported that adult *Drosophila* develop tolerance after a single ethanol exposure (Scholz et al., 2000, Berger et al., 2004). Flies upon exposure to ethanol vapour became hyperactive, lose postural control and eventually sedated (Moore et al., 1998; Singh and Heberlien, 2000). The sensitivity of a population of flies to ethanol can be measured using the sedation assay, a paradigm that quantifies ethanol-induced sedative effects of ethanol (Wen et al., 2005). Briefly, flies are introduced into a jar with a swab soaked with ethanol of a particular concentration. As flies became intoxicated with ethanol, they display hyperactivity before falling to the bottom of the jar and later become sedated. At 50% ethanol concentration (being the standard ethanol concentration used in this study, see Figure 4.5.2 of Chapter 4) wild-type flies reproducibly become sedated with a mean sedation time of ~54 min (Figure 5.1)

To determine whether rapid tolerance could be measured in the sedation assay, flies initially tested in the sedation assay as previously described, were collected in vials containing fresh food and allowed to recover in a humidified room at 18°C before re-testing in the sedation paradigm. The second exposure in the sedation assay (MST2) was initiated exactly 4 h after the start of the first exposure. The 4hr interval was chosen not only to ensure that flies had fully recovered from the sedative effect of ethanol but also to ensure they had completely metabolised all ethanol absorbed and had had time to rehydrate and feed (Scholz et al., 2000; Hancock, 2005, Chapter 3). Tolerance was calculated based on the percentage difference in MST between these two exposures (Figure 5.1). It was found that wild-type flies were more resistant to the second exposure displaying a MST of 71±4 % -an increase of nearly 18 min from the MST of their first exposure. In this

case, tolerance is defined as the relative increase in MST between the first and the second exposure, which for wild-type flies correspond to 33% under our standard experimental conditions.

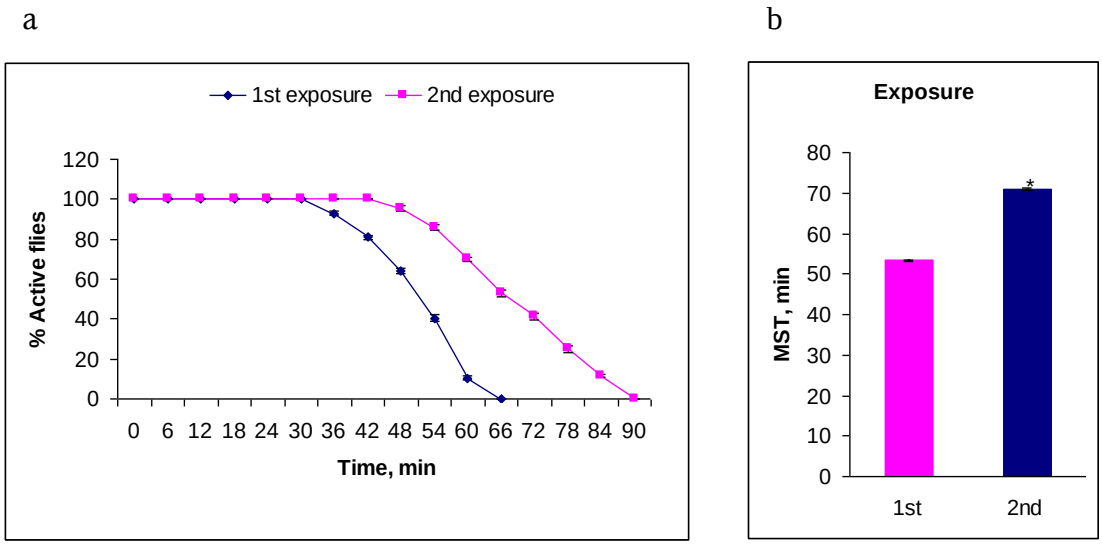


Figure 5.1 | Ethanol tolerance measured in the sedation assay

The sensitivity to ethanol vapour of a population of 20 flies was measured in the sedation assay, a simple but highly efficient technique that measures the duration of fly activity after exposure to a sedative dose of ethanol. At 50% standard ethanol concentration, wild-type flies become sedated with a curve (a) and a mean sedation time (MST) of 53.3 ± 0.3 min (b). When these flies were reintroduced into the vial 4 h after the first exposure, their sedation profile shifted to the right (a) and their new MST was 71.0 ± 0.4 min, which corresponds to a 33.0 ± 1.0% increase in resistance. Error bars represent standard error of the mean (SEM). N=20, $p<0.0001$. In all figures n correspond to the number of experiments, not the number of flies.

5.3.1 Kinetics of Rapid Tolerance Development and Decay

The kinetics of tolerance development has been previously described using the inebriometer (Scholz et al., 2000). Here, to determine the kinetics of tolerance decay using our sedation assay protocol, we exposed flies in the sedation jar twice at various time intervals (Figure 5.2) and quantified their tolerance acquisition at the appropriate time. Consistent with a previous characterisation of rapid tolerance using the inebriometer (Scholz et al., 2000), it was observed that the kinetics of tolerance dissipation was biphasic, suggesting the involvement of two different processes or mechanisms. Maximal tolerance, a 48.8 ± 1.9 % increase in MST, was achieved within a 2 h interval. Tolerance then decreased quickly, reaching ~12%

after 8 h. It then decayed more slowly and was still detectable 24 h after the first exposure, but had disappeared by 36 h (Figure 5.2). Thus, rapid tolerance measured with the sedation assay is very similar to that previously measured with the inebriometer, an assay that measures the fly's postural control on exposure an intoxicating dose of ethanol.

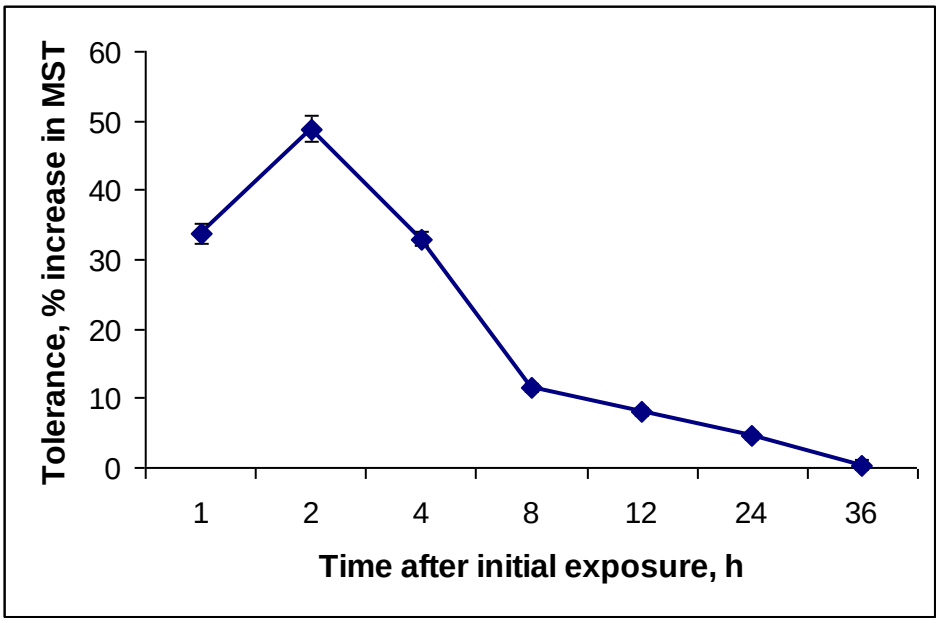


Figure 5.2| Kinetics of Tolerance acquisition and decay

Flies were exposed to ethanol once and then re-exposed after various time intervals. Tolerance was calculated for each set of flies as the percent increase in mean sedation time (MST) between the second and the first exposures. N= 20 (0 h and 4 h); n=6-10 (all other time points); error bars represents SEM.

5.3.2 Flies lacking octopamine showed reduced rapid tolerance in sedation assay

Octopamine is a phenolamine structurally related to the catecholamine norepinephrine in vertebrates (Certel et al., 2007). It acts as a neurohormone, neuromodulator, and neurotransmitter that functions in many processes equivalent to those using norepinephrine in vertebrates (reviewed in Roeder, 1999). Recently, in flies octopamine was demonstrated to play a role in modulating the choice between courtship and aggression in male flies (Certel et al., 2007), to function in

appetitive associative learning in flies (Schwaerzel et al., 2003); and in ethanol tolerance development (Scholz et al., 2000; Berger et al., 2004)

Tbh^{nm18} mutant flies defective for the octopamine biosynthetic enzyme Tbh were reported to display impaired rapid tolerance development using the inberiometer assay (Scholz et al., 2000; Berger et al., 2004). To investigate whether octopamine might also function in rapid tolerance in the sedation assay, the *Tbh*^{nm18} mutant flies were tested for ethanol tolerance in the tolerance paradigm. Flies were pre-tested in the sedation assay and were then re-assayed in the same paradigm after 4 h. Rapid tolerance was quantified on the basis of MSTs of first exposure and second exposure (Figure 5.3). As previously described, the *Tbh*^{nm18} mutant showed a marked decrease in ethanol tolerance compared to the wild type control (Figure 5.3), thus validating our tolerance protocol. On the basis of this finding, the mechanism governing rapid tolerance is at least in some part the same in both sedation and inebriometer assays.

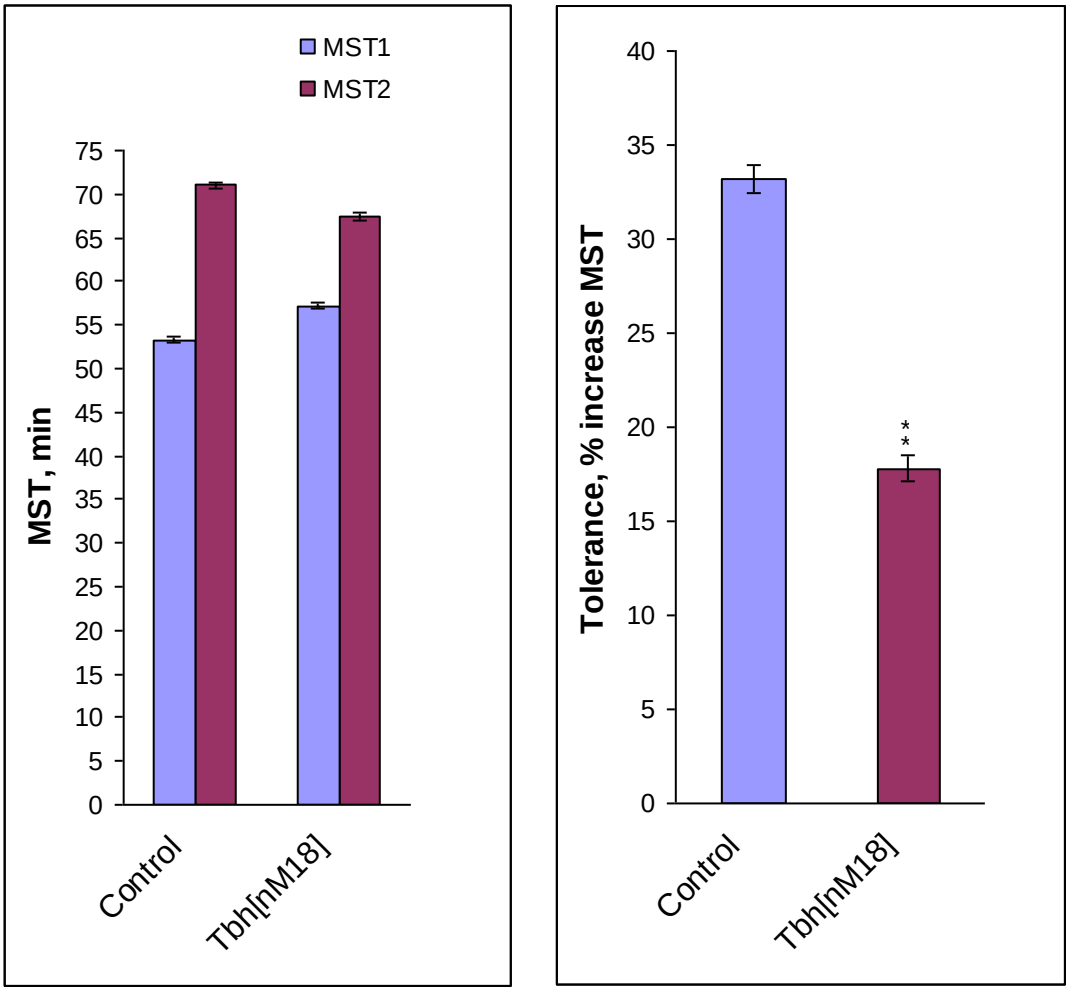


Figure 5.3 | Role for octopamine in rapid tolerance in sedation assay.

Tbh^{nm18} flies that carry a loss-of-function mutation in the gene encoding tyramine ß-hydroxylase (Tbh) and are thus unable to synthesise octopamine were tested for rapid ethanol tolerance development in the sedation assay. (A) *Tbh* flies are slightly more resistant than the wild-type control in the sedation assay, and (B) showed significantly reduced tolerance when tested 4h later, n=5, $p<0.00001$). Error bars represents SEM.

5.4 Ethanol tolerance of mutant flies

To gain an insight into molecules and pathways involved in tolerance development, rapid tolerance of the 4 selected candidate genes was carried out. Each line was first assayed in the sedation paradigm, given sufficient time (4 h) to recover and metabolise the ethanol, and then re-tested in the sedation assay. MSTs were determined for the first and second exposure of flies (denoted MST1 and MST2, respectively). To evaluate tolerance of these mutants, tolerance values were calculated as the increase in MST of flies in the first exposure to ethanol relative to the second exposure.

Several mutant alleles of the four genes (excluding controls) were tested for tolerance, and values ranged from a low of 0.3±0.4% ($hsp26^{EY10556}$) to a high of 59.1±2.1% (hiw^{ND8} (F)) (Figure 5.4, Table 5.1). The mean rapid tolerance of the control strain was 53.3±1.0% (for male) or 52.6±2.0% (for female).

A student's paired t-test assuming equal variance was employed and used to test for significance in alterations of the individual genes compared with the control in a pair wise manner. $mbf1^{2}$, and 2 alleles of *hsp83* ($hsp83^{08445}$ and $hsp83^{e6A}$) (F) showed reduced ethanol tolerance, 2 alleles of *hsp26* ($hsp26^{EY10556}$ and $hsp26^{KG02786}$) virtually abolished tolerance (Figure 5.4, Table 5.1). The 3 mutant alleles of *hiw* that were tested (hiw^{ND8}, hiw^{EP1308} and hiw^{EP1305}) did not exhibit any significant alterations in ethanol tolerance compared with the wild-type control flies. However, 2 of the female *hiw* mutants, hiw^{ND8} (F) and hiw^{EP1308} (F) displayed enhanced ethanol tolerance (Table 5.1, Figure 5.4).

Gene	Mutant	MST1, min. ±SEM, (n)	MST2, min. ±SEM, (n)	Rapid tolerance,±SEM
Wild-type		53.3 ± 0.3 (n=20)	71.0 ± 0.4 (n=20)	33.0 ± 1.0
Wild-type (F)		52.6 ± 0.7 (n=6)	69.7 ± 0.5 (n=6)	32.5 ± 2.0
mbf1	$mbf1^{2}$	39.5 ± 0.7 (n=6)	44.9 ± 0.2 (n=6)	13.6 ± 2.0
	$mbf1^{+}$	51.2 ± 0.6 (n=6)	59.4 ± 0.2 (n=6)	16.1 ± 1.5
Hsp83	$hsp83^{08445}$(F)	74.5 ± 2.3 (n=8)	96.0 ± 0.1 (n=6)	28.8 ± 4.0
	$hsp83^{08445}TM3$(F)	50.1 ± 0.6 (n=6)	67.8 ± 0.5 (n=6)	35.5 ± 2.0
	$hsp83^{e6A}$/+(F)	63.4 ± 0.3 (n=6)	73.2 ± 0.2 (n=6)	15.5 ± 0.7
Hsp26	$hsp26^{EY10556}$	54.0 ± 0.2 (n=6)	54.2 ± 0.1 (n=6)	0.3 ± 0.4
	$hsp26^{EY10556}$/+	53.1 ± 0.2 (n=6)	67.8 ± 0.3 (n=6)	27.7 ± 0.6
	$hsp26^{EY10556}$(F)	62.9 ± 0.4 (n=6)	61.8 ± 0.3 (n=6)	-1.8 ± 0.8
	$hsp26^{KG02786}$	53.1 ± 0.4 (n=6)	55.4 ± 0.3 (n=6)	4.3 ± 1.0
Hiw	hiw^{EP1308}	39.5 ± 0.3 (n=8)	52.7 ± 0.4 (n=6)	33.6 ± 1.5
	hiw^{EP1308}(F)	39.8 ± 0.2 (n=6)	55.1 ± 0.3 (n=6)	38.5 ± 0.9
	hiw^{EP1305}	40.3 ± 0.2 (n=6)	53.8 ± 0.2 (n=6)	33.4 ± 0.9
	hiw^{EP1305}(F)	40.3 ± 0.2 (n=6)	54.5 ± 0.3 (n=6)	35.2 ± 1.0
	hiw^{ND8}	41.8 ± 0.3 (n=6)	56.4 ± 0.4 (n=5)	34.9 ± 1.4
	hiw^{ND8}(F)	41.1 ± 0.2 (n=6)	65.4 ± 0.8 (n=5)	59.1 ± 2.1

Table 5.1 | Rapid ethanol tolerance of the four genes tested

Ethanol tolerance was quantified in the sedation paradigm (Materials and Methods). All values are mean ± SEM. For each genotype, n=number of experiments and not the number of flies. F= indicates female flies.

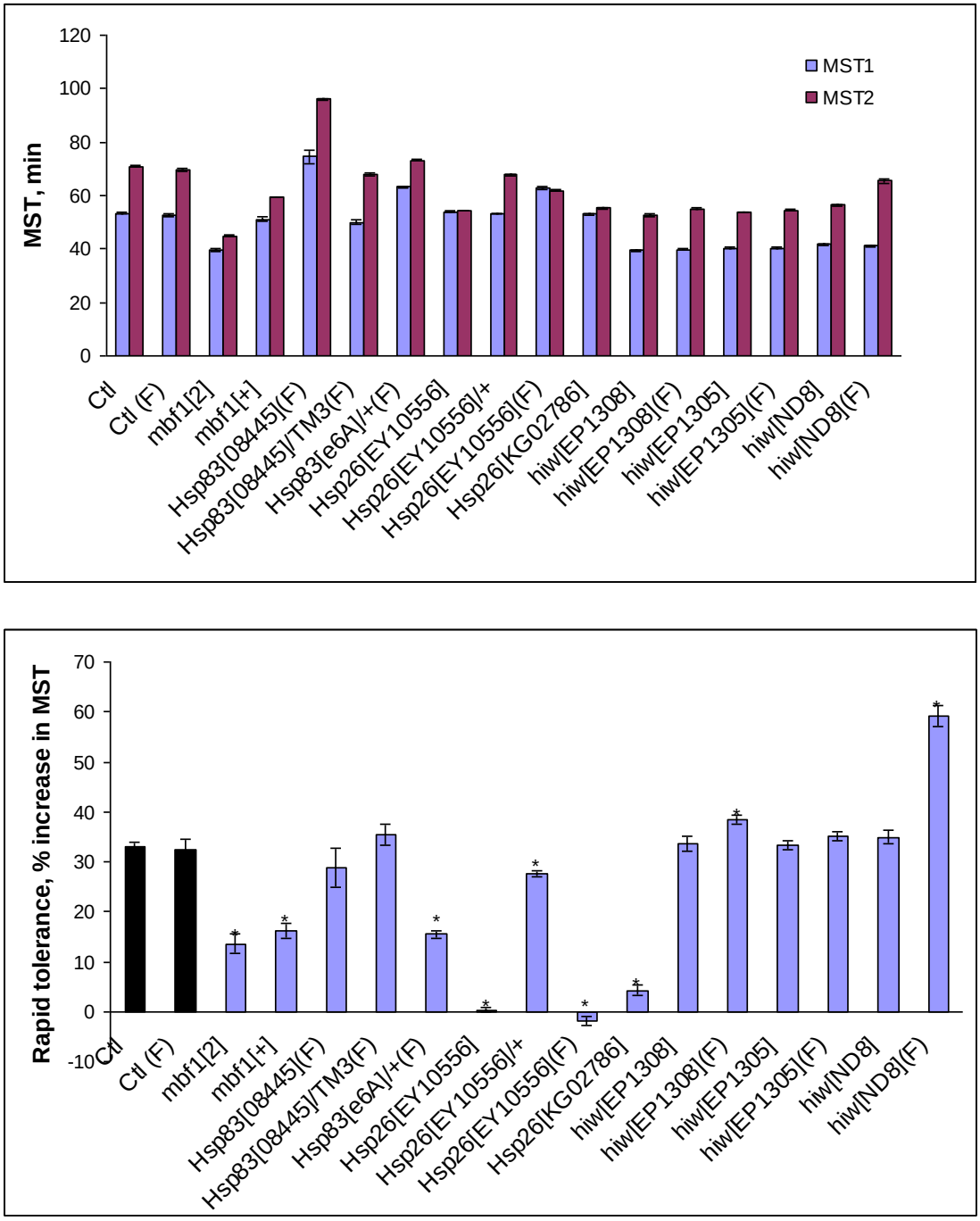


Figure 5.4| Rapid ethanol tolerance of all the mutants tested.

Flies were pre-tested in the sedation assay, allowed to recover for 4.0 hrs and later re-assayed in the same paradigm. (A) MET and (B) Rapid tolerance values for these mutants. Asterisk denotes $p<0.001$ for each mutant versus wild-type control. Error bars represent SEM. For the number of experiments carried out on each mutant refer to Table 5.1.

5.4.1 Mutations affecting the *hsp26* gene abolish ethanol tolerance

To ascertain a possible role for *heat shock protein 26* gene (the most highly up-regulated gene tested) in ethanol tolerance, two mutant alleles carrying different P-element insertion in the same position near the *hsp26* gene were tested in the tolerance paradigm. Whereas the ethanol sensitivities of these male alleles were normal in the sedation assay (Chapter 4, Figure 4.8), these flies displayed virtually abolished tolerance when tested 4 h after the initial ethanol exposure (Figure 5.5) The behaviour of these alleles cannot be explained by an alteration in the kinetics of tolerance acquisition as a similar marked defect in tolerance was observed when these two alleles were exposed to ethanol using protocols earlier shown to produce maximal tolerance in the sedation assay (Figure 5.6). Both male and female $hsp26^{EY10556}$ flies behaved in the same manner. Male flies heterozygous for the $hsp26^{EY10556}$ mutant were also tested for development of rapid tolerance. The $hsp26^{EY10556}$ heterozygous males displayed ethanol tolerance that is 75% of wild-type level suggesting that tolerance is sensitive to the dosage of *hsp26*. Alternatively, this may be an effect of genetic background. To eliminate this latter possibility, heterozygotes of the *KG02786* allele of *hsp26* should be tested. Nevertheless, the two independently isolated *hsp26* alleles in their homozygous state exhibited very similar ethanol related behaviours: both showed normal sensitivity and virtually abolished ethanol tolerance development. This indicates the specificity of this behaviour to the *hsp26* gene and also rules out any effects due to genetic background at least in the homozygous state.

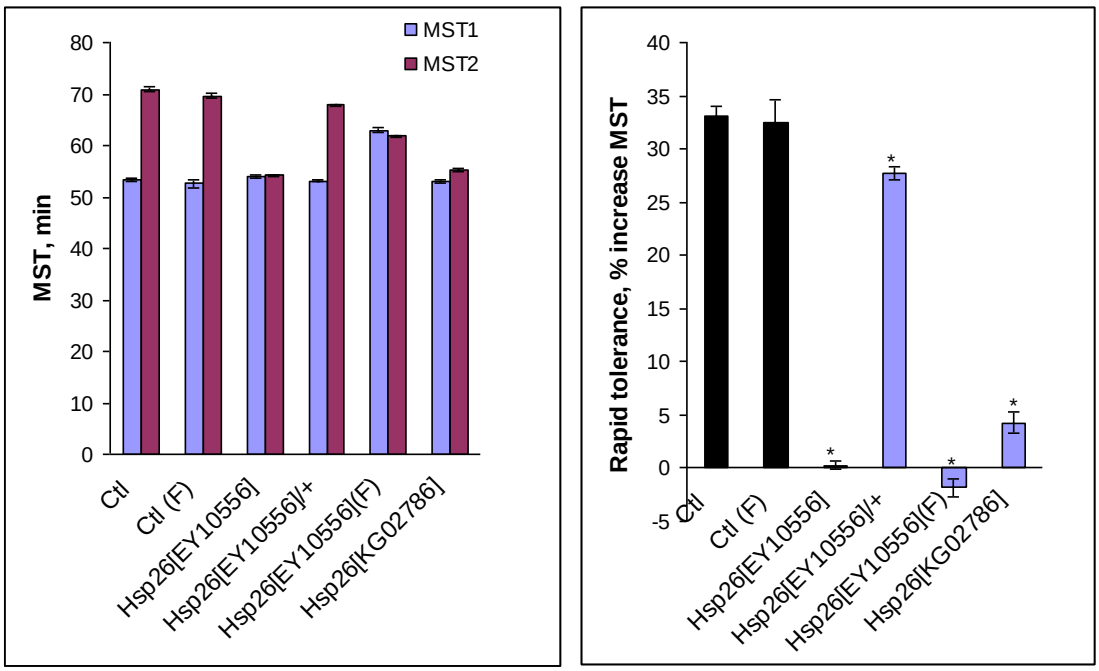


Figure 5.5| Effect of *hsp26* on ethanol tolerance

Two independently isolated *hsp26* alleles carrying P-element insertions in the same position were tested for tolerance in the sedation paradigm. (A) $hsp26^{EY10556}$ and $hsp26^{KG02786}$ male flies were indistinguishable from wild-type control in the first sedation exposure, but $hsp26^{EY10556}$ females showed a significantly reduced sensitivity in the first exposure compared to wild-type control female flies. n=6, $^{*}p<0.0001$. (B) Both male and female flies from the two *hsp26* alleles were however defective in rapid tolerance quantified in the second sedation exposure measured 4 h after the first exposure, n=6, $^{*}p<0.0001$. Error bars represents SEM.

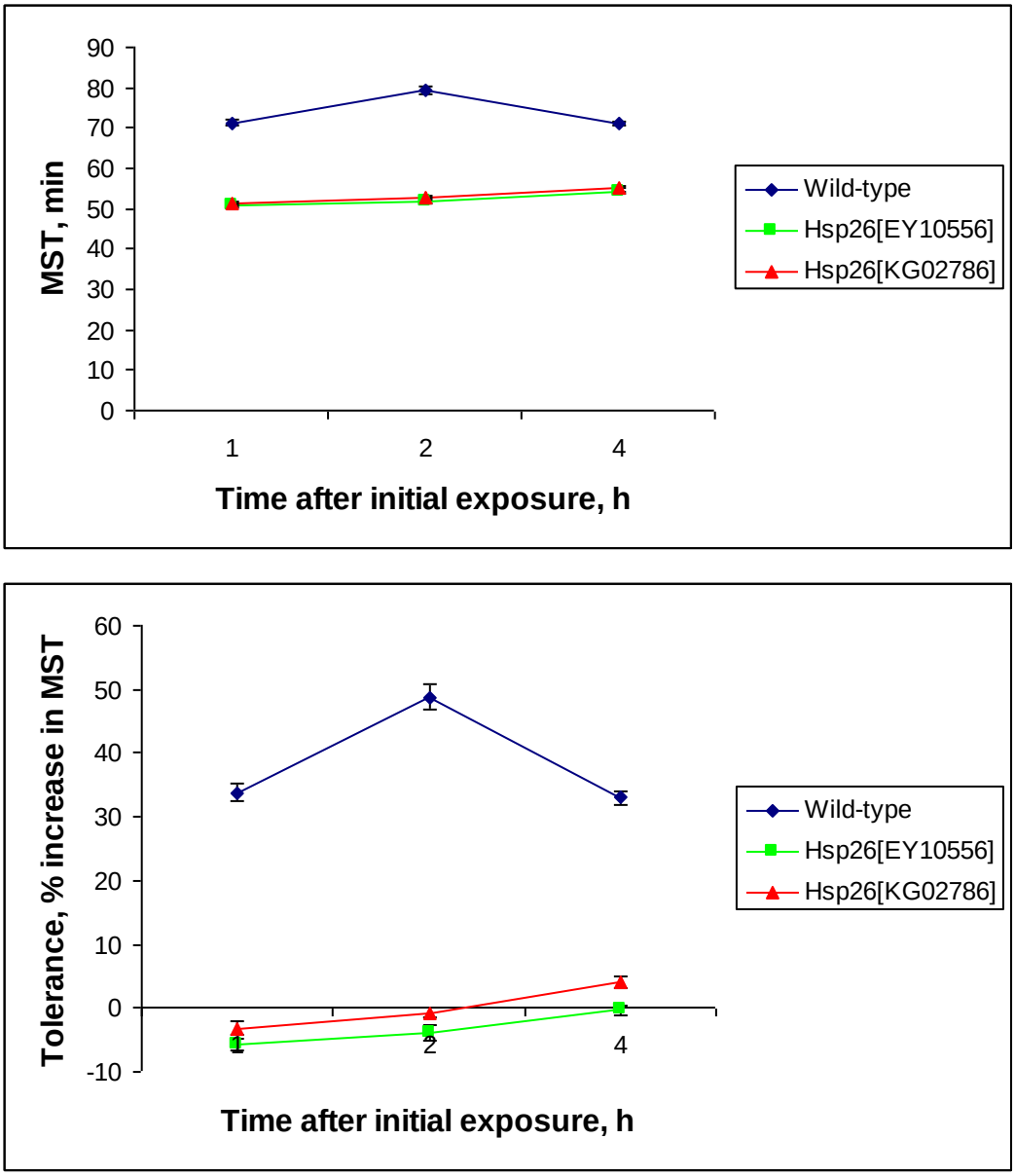


Figure 5.6| Kinetics of Tolerance behaviour in *hsp26* mutant flies

Flies were exposed to ethanol once and then tested in the sedation assay after the three time intervals shown. Tolerance was calculated for each set of flies as the percent increase in mean sedation time (MST) between the second and the first exposures. It shows a marked profile difference in the kinetics of dissipation between the wild-type and the two *hsp26* mutant flies (*hsp26*^{EY10556} and *hsp26*^{KG02786}). N= 6-20 (0 h and 4 h); n=4-10 (1 h and 2 h). Error bars represents SEM.

Exposure to an acute dose of ethanol induces a stress response (Alexandre et al., 2001). We wished to test whether the stress pathway(s) induced by ethanol are linked to, or overlap with, stress pathways induced by other stressors. Hsp26 encodes a cytoplasmic protein involved in the response to several forms of stress, including heat shock (Jiménez-Martí et al., 2009). To assess whether prior heat shock of flies might mediate ethanol tolerance in the sedation assay, and determine whether a heat pulse could mimic the tolerance effects seen in the *hsp26* alleles, we tested both the wild-type control and *hsp26* flies for ethanol tolerance using the heat shock protocol (see section 2.3.5 of the Materials and Methods). Heat exposure of wild-type flies (38°C for 5 min) led to a 42 % increase in MST when measured in the sedation assay 4 h later. However, the two *hsp26* alleles displayed 10 % and 9 % increase in MST when treated with the same protocol. Thus two *hsp26* genotypes displayed a substantially reduced level of tolerance development compared with the control flies ($P < 0.001$, Figure 5.7). Given that *hsp26* alleles are deficient in both forms of tolerance, indicate that the tolerance produced by EtOH and heat overlaps. In addition, in a *hangover* gene like-manner, *hsp26* flies retain some capacity for developing tolerance prior to heat shock, suggesting that other pathways are also involved (Scholz et al., 2005).

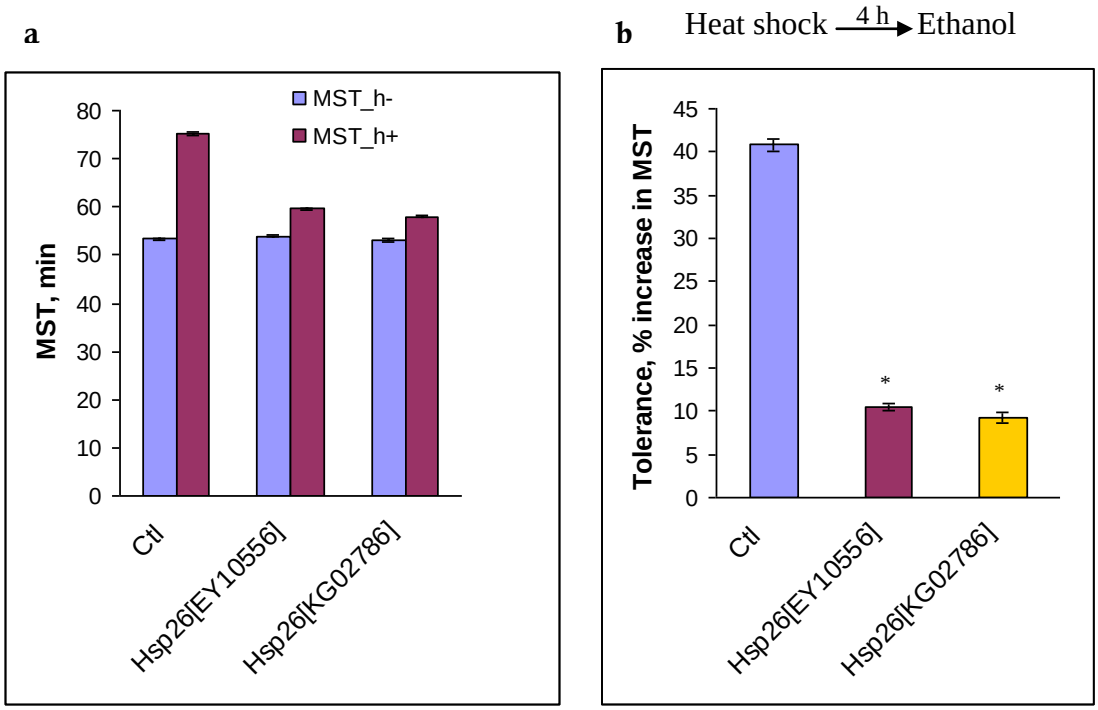


Figure 5.7| *hsp26* ethanol tolerance after heat shock.

Heat– ethanol cross-tolerance of wild-type and *hsp26* flies (section 2.3.5 of the Materials and Methods). (a) *hsp26* flies are indistinguishable from wild-type control in the first sedation exposure without the heat shock. (b) *hsp26* mutant flies however showed significantly reduced tolerance development in the sedation assay following heat pulse exposure compared to the control (wild-type) flies. n=6, $^{*}p<0.0001$. MST_h^{-} and MST_h^{+} denote MST obtained from flies without and after heat shock respectively. Error bars represent SEM.

Previous studies have reported two pathways involved in fly's ethanol tolerance development: the stress pathway defined by *hangover* gene (*hang*) and the octopaminergic pathway defined by a *Tbh* gene (Scholz et al., 2005). To test which of these molecular pathways might be mediated by *hsp26* gene (or vice versa) required that flies lacking a combination of *hang* and *hsp26* gene product or *Tbh* and *hsp26* gene product be tested for ethanol tolerance using a genetic epistasis test. Consequently, flies hemizygous for *Tbh* gene and heterozygous for *hsp26* gene were generated and tested for rapid tolerance. Ethanol tolerance is completely restored in Tbh^{nm18}/Y; +/+; $Hsp26^{EY10556}$/+ flies (Figure 5.8). As $hsp26^{EY10556}$/+ flies have tolerance significantly different from the control flies (Figure 5.8), these data

suggest that two parallel molecular pathways are involved and as such the tolerance defect seen in *hsp26* flies does not involve octopaminergic systems.

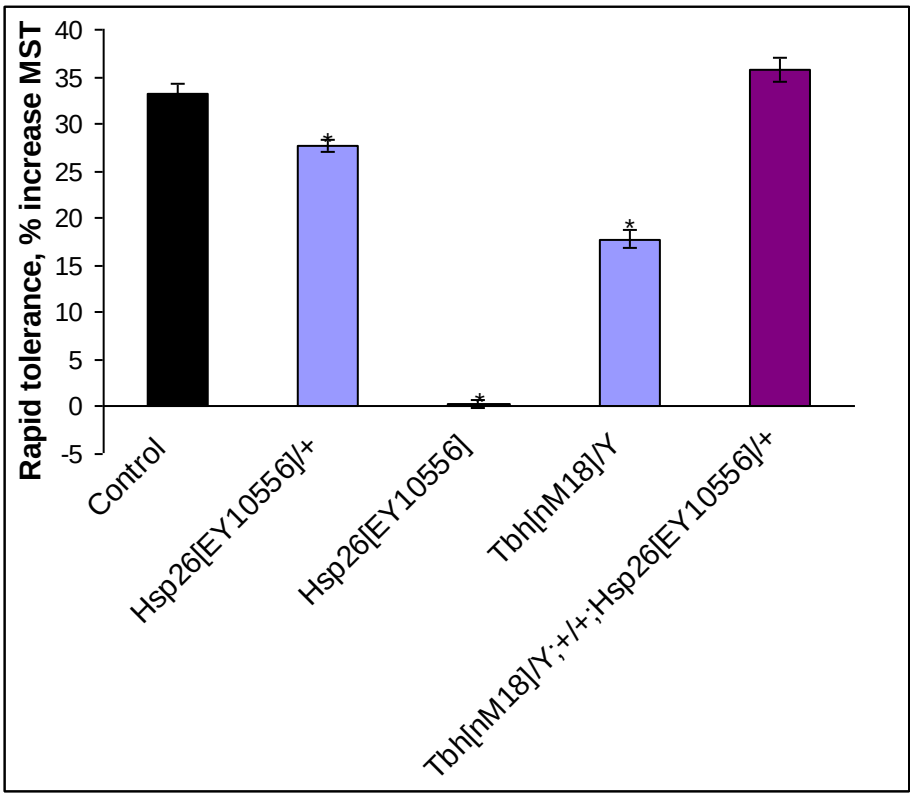


Figure 5.8| Tolerance is restored in *hsp26* and *Tbh* double mutant flies

Tbh^{nm18}/Y; +/+; $hsp26^{EY10556}/+$ flies were tested for tolerance in the sedation assay. Tbh^{nm18} complemented the ethanol tolerance of $hsp26^{EY10556}$ (n=5). Error bars represent SEM.

5.4.2 Mutations affecting the *hsp83* gene show reduced ethanol tolerance

To ascertain a possible role for *Drosophila* Hsp90 protein in ethanol tolerance, a P-element-induced mutant for Hsp90 protein, the *scratch* ($hsp83^{08445}$ allele) was tested in the sedation assay. Whereas these flies have decreased ethanol sensitivity (Chapter 4, Figure 4.9), they developed marginally significantly reduced ethanol tolerance, measured 4 h after the initial ethanol exposure compared with either wild-type control or $hsp83^{08445}/TM3$ flies (P=0.04, Figure 5.9). The $hsp83^{08445}/TM3$ flies displayed ethanol tolerance that was indistinguishable from the wild-type control when measured in the sedation paradigm (Figure 5.9). $Hsp83^{08445}$ is a viable allele of *hsp83*. However, a lethal allele $hsp83^{e6A}$, when tested over a wild-type chromosome

(*hsp83*^{e6A}/+) displayed a statistically significantly reduced ethanol tolerance compared to the wild-type control (P<0.0001, Figure 5.9). The reduced ethanol tolerance phenotype of *hsp83*^{e6A} heterozygotes was significantly more severe than that of *hsp83*^{08445} homozygotes (P<0.0001). The Hsp90 mutant, *hsp83*^{08445} has a P-element inserted in the 5' intron of the gene and leads to a small reduction in Hsp90 protein (Yue et al., 1999). The *hsp83*^{e6A} mutation on the other hand, is most likely a loss-of-function mutation or a very strong hypomorph as it is an EMS-induced mutation affecting the coding region of an exon.

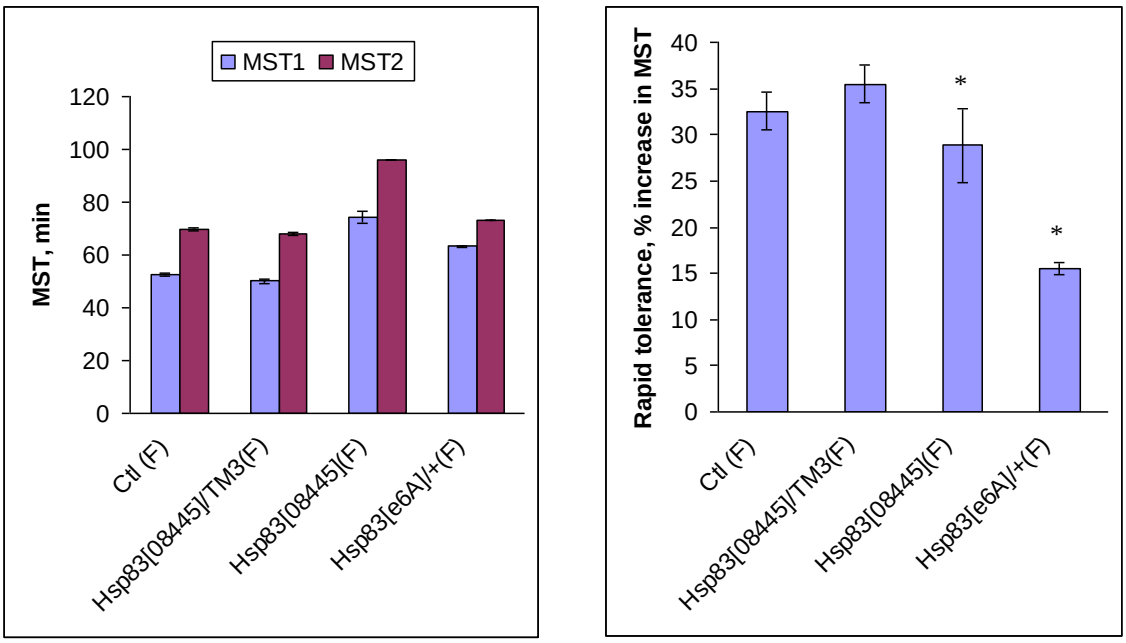


Figure 5.9 | Effect of *hsp83* on ethanol tolerance

(A) Left panel shows mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol (second exposure, purple bars). (B) Right panels shows development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. While the two *hsp83* mutant flies display significantly reduced sensitivity to ethanol on first exposure in (A), they also show a significantly reduced tolerance ($^{*}p<0.05$) compared with the wild-type controls in (B). n=6-8 and error bars represent SEM.

5.4.3 Mutations affecting the *mbf1* gene lead to reduced tolerance

The loss of *mbf1* gene has been shown to affect the fly's sensitivity to ethanol (Chapter 4, Figure 4.10). To test the possibility that the loss of *mbf1* affects ethanol tolerance in *Drosophila*, a stock homozygous for the $mbf1^2$ mutation and another stock with a genomic fragment containing the *mbf1* gene in an $mbf1^2$ mutant background (P*[mbf1⁺]; mbf1²*) were tested twice at 4 h interval in the sedation assay. It was observed that $mbf1^2$ animals showed reduced ethanol tolerance compared with the control strain ($P<0.01$, Figure 5.10). However, whereas ethanol sensitivity of the control strain, P*[mbf1⁺]; mbf1²* flies were normal, this strain displayed substantially reduced tolerance compared with wild-type flies ($P<0.001$, Figure 5.10). P*[mbf1⁺]; mbf1²* flies have previously been shown to rescue a number of phenotypes (Jindra et al., 2004), the behaviour displayed in the tolerance assay suggests that it is unable to rescue the tolerance phenotype to a wild-type level. A possibility is that the level and/or temporal expression of the inserted *mbf1* gene is not compatible with the acquisition of wild-type levels of tolerance.

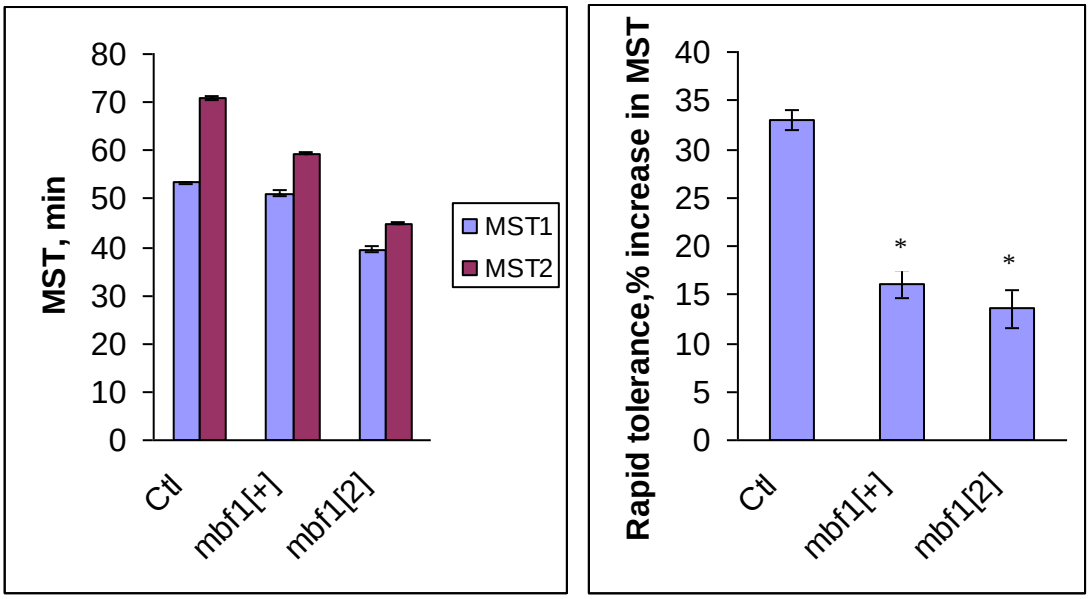


Figure 5.10| Effect of *mbf1* on ethanol tolerance

(A) Left panel shows mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol (second exposure, purple bars). (B) Right panels shows development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. $mbf1^2$ mutants show significantly reduced tolerance compared to wild-type control, $^*p<0.00001$, but only marginally significantly reduced compared to mbf1[⁺] control, $^*p<0.04$). n=6, error bars represent SEM.

5.4.4 Mutations affecting the *hiw* gene show sex-specific effects on tolerance

To determine whether the *hiw* gene is implicated in ethanol tolerance, three alleles of *hiw* were tested in the sedation assay protocol. Whereas the initial ethanol sensitivity was enhanced in all the three alleles (Chapter 4, Figure 4.6), two of the alleles displayed sex-specific effects in ethanol tolerance (Figure 5.11, Table 5.1).

When the male *hiw* mutants for all the three alleles were assayed for tolerance, they did not exhibit any significant alterations in ethanol tolerance. Conversely, two of the three female *hiw* alleles display statistically significant increase in ethanol tolerance compared with control flies ($P< 0.001$, Figure 5.11). This effect is specific to ethanol tolerance, as mutations in both sexes have been shown to cause an increase in ethanol sensitivity (Chapter 4, Figure 4.6). The enhanced tolerance effect is highly pronounced in the hiw^{ND8} female allele with a percentage increase in tolerance of 59.1 ± 2.1 compared with 32.5 ± 2.0% for wild-type flies. The reason for this overt behaviour is not known.

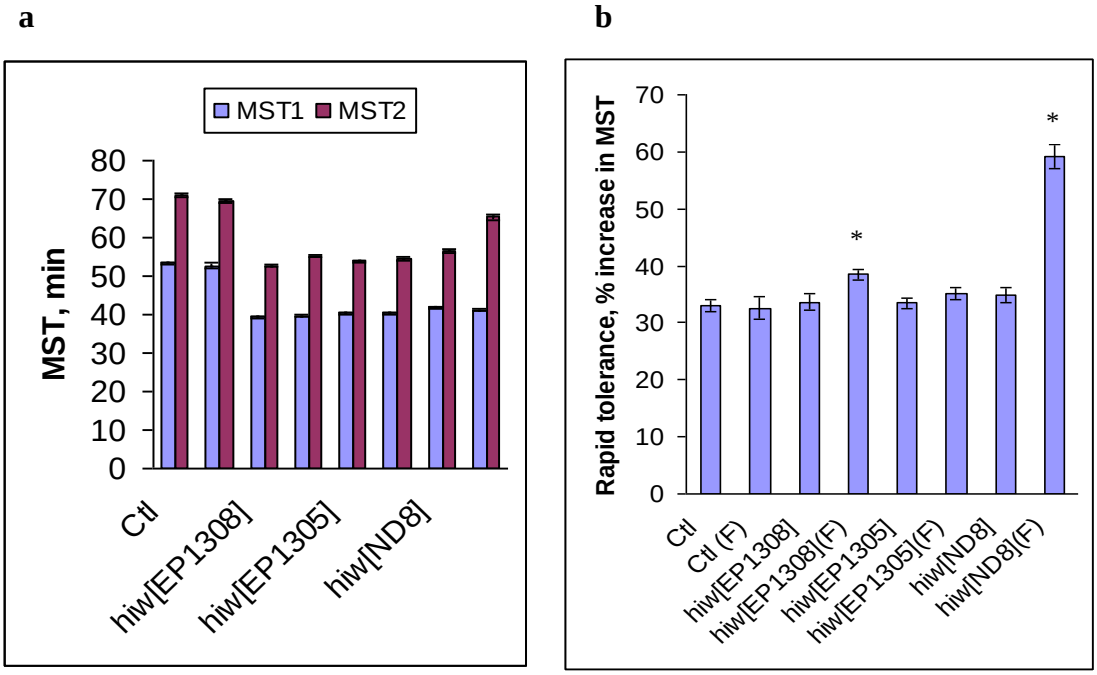


Figure 5.11 | Effect of *hiw* on ethanol tolerance

(a) Left panel shows mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol (second exposure, purple bars). (b) Right panels show development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. While the male *hiw* mutants display indistinguishable ethanol tolerance from the wild-type control, two female *hiw* mutants of hiw^{EP1308} and hiw^{ND8} show significantly increased tolerance ($^{*}p<0.001$) compared with the wild-type controls in (B). n=5-8 and error bars represent SEM.

5.5 Ethanol absorption and metabolism

One possible explanation for the altered ethanol phenotypes observed for the mutants tested in this study is that the modulation of expression of all the tested genes is an adaptive response of the fly nervous system to compensate for at least some of the sedating effects of ethanol, and this change in expression is at least partially responsible for the altered sensitivity and rapid tolerance to ethanol. However, it is also possible that these mutants exhibit altered ethanol phenotypes as a result of differences in their ethanol absorption and/ metabolism when compared to a wild-type strain.

To determine whether there was altered ethanol metabolism in flies, the ethanol concentration in fly extracts prepared from *mbf1*^{2}, *hsp26*^{EY10556}, *hiw*^{ND8}, *hsp83*^{08445} or wild-type flies exposed to constant ethanol vapour (100%) for 12 min and allowed to recover for 0, 1, 2, 3, or 4 h was measured. All protocols and parameters are in section 2.4 of Chapter 2. The ethanol concentration in extracts of each of the mutant fly was indistinguishable from that of the wild-type control flies at all time points (Figures 5.12). As shown in the Figure, the results reveal a very similar rate of ethanol clearance for all genotypes. Therefore, the altered ethanol phenotypes of these mutants were more likely due to pharmacodynamic changes, and not pharmacokinetic changes. The results also give an ethanol content of about 44mM in wild-type flies immediately after sedation. This corresponds to about 0.20% in human blood alcohol concentration (BAC) which causes severe loss in sensory stimuli and loss of consciousness (NIAAA, 2003).

Heberlein and colleagues at the University of California, San Francisco have reported an ethanol concentration of 32 mM in fly extracts prepared immediately after a 20 min exposure in the inebriometer (Scholz et al., 2000) when some flies may not have been completely sedated. Conversely, in our protocol, complete sedation was observed when flies were treated with 100 % EtOH for 12 min in the recovery assay. This in fact, could account for the higher ethanol content seen in fly extracts prepared immediately after this 12 min exposure when compared with that of the inebriometer.

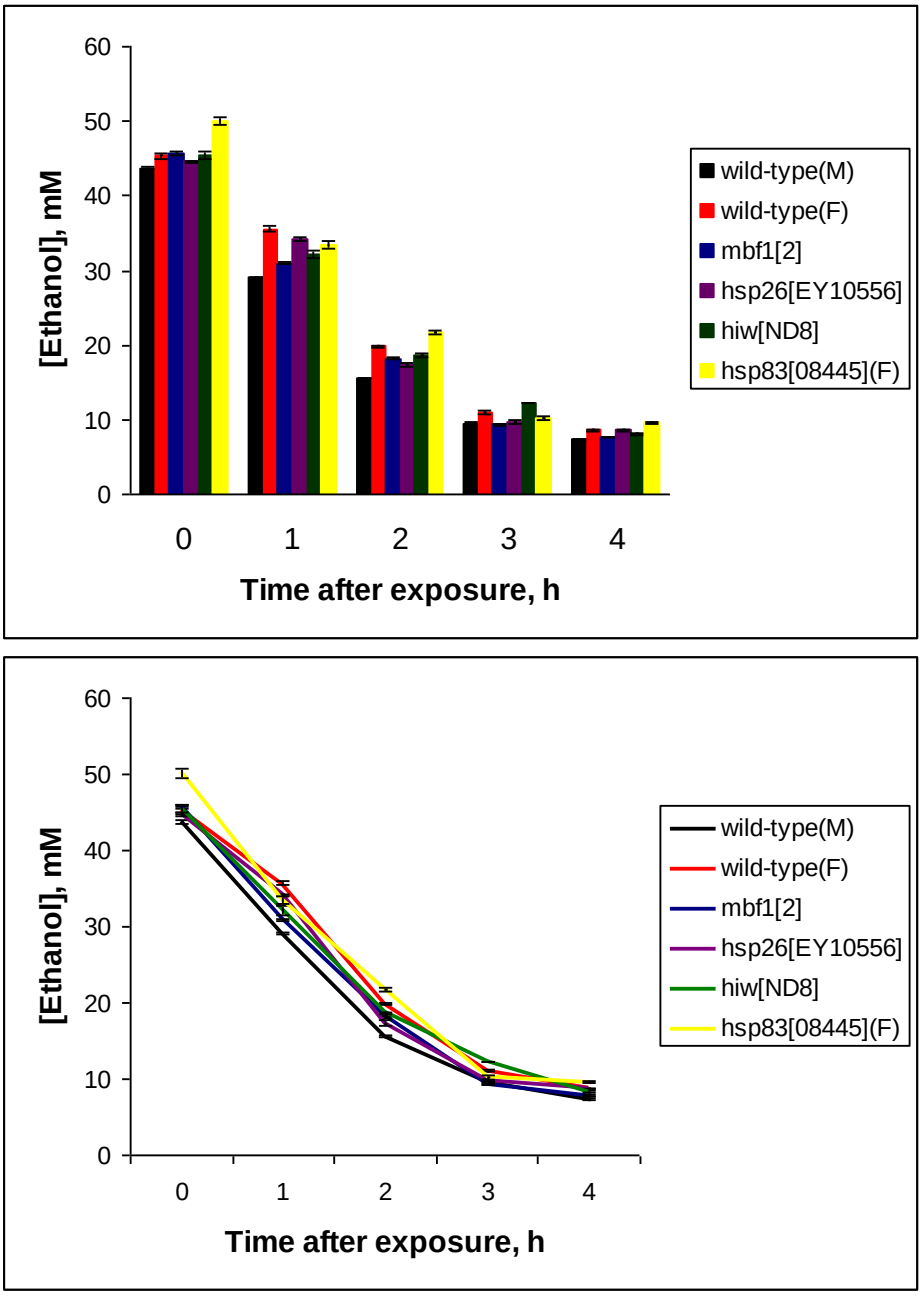


Figure 5.12 | Ethanol concentrations in flies after their ethanol treatment

Ethanol concentrations after 12 min exposure to ethanol vapour is shown. No significant differences were seen between each mutant and the wild-type control (Student's t test; n=3). A One-way ANOVA analysis of all the different groups specified showed no significant differences across genotypes for each time point ($P = 0.55$ for 0 h; $P = 0.29$ for 1 h; $P = 0.32$ for 2 h; $P = 0.82$ for 3 h and $P = 0.32$ for 4 h). However, a One-way ANOVA with post hoc Newman Keuls pair-wise planned comparisons across the time group revealed significant differences between 4 h and 0 h; 4 h and 1 h; 4 h and 2 h; 3 h and 0 h; 3 h and 1 h; 3 h and 2 h; 2 h and 0 h; 2 h and 1 h; and 1 h and 0 h ($P < 0.001$ for all comparisons). However, no significant difference was seen between 4 h and 3 h ($P > 0.05$).

5.6 Discussion

Drosophila is a suitable model system in which to study molecular mechanisms that regulate various aspects of ethanol tolerance (Scholz et al., 2000). To obtain an understanding of the molecular mechanisms underlying tolerance to ethanol action, we established one independent assay to measure tolerance to the sedative effects of ethanol in *Drosophila*. Using this assay, rapid tolerance, which is induced by exposure of flies to a single moderately high ethanol dose, was quantified as a reduction in sensitivity observed upon administration of a second dose of ethanol delivered at a time when the first dose is completely metabolised (Crabbe et al., 1979). Previous studies have examined and characterised rapid tolerance in *Drosophila* using various assays (Scholz et al., 2000; Dzitoyeva et al., 2003; Berger et al., 2004; Urizar et al., 2007; Cowmeadow et al., 2005; Cowmeadow et al., 2006). For instance, Scholz et al., (2000) have characterised rapid tolerance using the inebriometer assay. However, none of these studies have characterised tolerance for the assay used in this investigation. In this study and using the sedation assay protocol, the quantitative aspects of ethanol tolerance in flies, such as the extent of maximal tolerance and the kinetics of its decay (dissipation) are similar to those previously described using the inebriometer in flies (Scholz et al. 2005). In addition, it was shown that octopaminergic systems earlier implicated as a component of rapid tolerance (Scholz et al., 2000) are also involved in impaired ethanol tolerance in this assay. Taken together, these data demonstrate that the sedation assay is a suitable paradigm in which to measure fly's tolerance to the sedative effects of ethanol.

It is however, important to contrast the tolerance assay used in this study with that of the inebriometer that measures the flies' postural control (Heberlein 2000). The tolerance paradigm differs in that it measures sedation in response to discrete doses of ethanol. The assay has been extended to measure the flies' acquired resistance or tolerance, to the effects of ethanol on sedation rather than on postural control and locomotion. The results indicate that these two assays may share overlapping mechanisms in their ability to measure flies' ethanol tolerance, since *Tbh* flies displayed reduced rapid ethanol tolerance in both assays (this study and Scholz et al., 2000).

Using the sedation assay, one mutant (*mbf1*^{2}) earlier implicated in enhanced ethanol sensitivity, displayed reduced ethanol tolerance. In line with this, *homer* mutant flies have previously been reported to display increased ethanol sensitivity and reduced ethanol tolerance (Urizar et al., 2007). Further, Berger et al., (2008) in a collection of 52 *Drosophila* long-term memory mutants, identified 8 mutants with reduced rapid tolerance out of which 4 mutants were shown to have increased ethanol sensitivity and one with decreased ethanol sensitivity. In experiments that identified 20 fly lines with an altered transcriptional response to acute ethanol, genes with decreased ethanol sensitivity most often showed increased tolerance. This same study identified 7 strains with enhanced ethanol tolerance rather than reduced tolerance (Morozova et al., 2006), suggesting that there are some overlaps between the mechanisms that underlie sensitivity to and tolerance of alcohol. However in this study there were two genes whose defects were specific to sensitivity or tolerance, indicating that these processes are at least partially distinct. Similarly flies lacking *octopamine* showed no change in ethanol sensitivity, yet showed reduced tolerance in inebriometer in the same manner as flies lacking *hang* gene product (Scholz et al., 2000, Scholz et al., 2005).

Multiple alleles were available for some of the genes that were tested. The two mutants, *hsp83*^{e6A} and *hsp83*^{08445} exhibited similar ethanol behaviours: both showed increased sensitivity and reduced tolerance. The 2 mutants, *Hsp26*^{EY10556} and *Hsp26*^{KG02786} are P-element insertions at the same site (www.flybase.org), and exhibited very similar ethanol-related behaviours: both abolished rapid ethanol tolerance and showed normal ethanol sensitivity. The mutants *hiw*^{EP1308}, *hiw*^{EP1305} and *hiw*^{ND8} earlier shown to have an increased ethanol sensitivity, displayed normal ethanol tolerance. While *hiw*^{ND8} is a loss of function mutation, *hiw*^{EP1308} and *hiw*^{EP1305} are caused by P element insertions in different positions within the gene (Wu et al., 2005). The fact that these behavioural responses were seen in more than one allele of the same stocks, greatly increases the likelihood that mutations in these genes, rather than unlinked second-site mutations, are responsible for the altered ethanol phenotypes.

This study has also showed a marked interaction between sex and ethanol treatment in two genes, *hsp26* and *hiw* with some changes of behaviour being restricted to females of *hsp26* and *hiw* respectively. Male *hsp26* flies showed normal ethanol sensitivity in the sedation assay whereas female flies displayed reduced sensitivity. Conversely, male *hiw* flies displayed normal ethanol tolerance whereas the female flies showed enhanced ethanol tolerance to the sedative effect of ethanol. This shows the involvement of highly complex regulatory mechanisms in both the sensitivity and tolerance of these genes to ethanol in the two sexes. Thus, the dynamics of ethanol-induced effects are very different in the two sexes, and might shed light on the numerous effects of sex differences found in *Drosophila* (Sørosen et al., 2007). Interestingly, evidence from epidemiological and clinical studies has shown notable sex differences in alcohol use and propensity for abuse and dependence (Vetter-O'Hagen et al., 2009). For example, differences between men and women in their sensitivity to a number of acute or chronic consequences of ethanol have been reported (Fillmore and Weafer, 2004, NIAAA, 2004). In rodents, mature females have been shown to display higher ethanol intake than their male counterparts (Lê *et al.*, 2001, Chester *et al.*, 2006).

Studies have shown that that ethanol can induce heat shock proteins (Alexandre et al., 2001, Pignataro et al., 2007). As their name suggests, these evolutionary conserved proteins were originally characterised on the basis of their strong induction by heat shock, but they are also induced by a number of chemical agents including ethanol (Piper et al., 1994). In my microarray studies, I showed in Chapter 3 that acute ethanol triggers the activation of a suite of such heat shock genes. Two of these genes, *hsp83* (an ATP-dependent chaperone) and *hsp26* (which belongs to a class of small ATP-independent heat shock proteins, sHsps) affect the fly's tolerance to ethanol in this study.

Hsp90 is an abundant and ubiquitous cellular protein that is indispensable for cell survival even under non-stressful conditions (Hendrick and Hertl, 1993). This protein has been shown to prevent the aggregation of chemically denatured or heat denatured proteins (Jakob et al., 1995). The increase expression of Hsp90 gene from the microarray and its involvement in acute ethanol response, therefore, strongly

suggests that this gene may be an important element in regulating protein stability while protecting the cells against the depressive effects of ethanol. Interestingly, the Hsp90 mutant flies mimic the behaviour displayed by Cyc loss--function flies by increasing their mortality rates upon sleep deprivation (Shaw et al., 2002). Activation of Hsp90 protein on the other hand was shown to rescue Cyc null mutants from the lethal effects of sleep deprivation (Shaw et al., 2002), indicating a role of Hsp90 in clock homeostasis/ regulation. Recently, an increase in the transcription of Hsp90 in cultured mouse cortical neurons exposed to an acute dose of ethanol has been shown (Pignataro et al., 2007). Thus, the implication of Hsp90 in the transcriptional regulation of acute ethanol-induced behaviour has, in itself, important implications for cellular responses to ethanol.

The *hsp26* gene was the gene most highly induced on exposure to acute doses of ethanol. Mutants of *hsp26* have been shown to abolish ethanol tolerance, and reduce heat induced tolerance to ethanol in this study. In yeast, the loss of the *hsp26* gene showed no overt heat sensitivity or thermotolerance but is involved in the stress tolerance of yeast during ethanol production (Sharma et al., 2001). *Hsp26* has also been shown in yeast to play an important role in pathways that defend cells against environmental stress and the types of protein misfolding seen in neurodegenerative diseases (Cashikar et al., 2005). Thus, the significance of Hsp26 protein as a chaperone and its functional regulation in the nervous system could provide new insights into the contribution of stress-induced signalling mechanisms in brain cells exposed to acute or chronic ethanol.

Hiw codes for an ubiquitin ligase and displays increased sensitivity, and normal / enhanced ethanol tolerance. Interestingly, an interaction between protein ubiquitination and stress pathway (specifically, sHsps pathway) has earlier been suggested (reviewed in Joanisse et al., 1998), indicating that sHsps may be involved in the modulation of protein ubiquitination activity. Ethanol has the tendency to destabilise the hydrophobic interactions of proteins which could lead to the interaction of Hsp chaperones with the destabilised substrate proteins and the concomitant activation of heat shock genes (Mager and Moradas-Ferreira, 1993). The upregulation of the heat shock pathway is, therefore, to minimise inappropriate

interactions between destabilised proteins and prevent their precipitation under ethanol stress (Treweek et al., 2000). One way through which they modulate the stability of these substrate proteins may be through the ubiquitin-proteasome regulatory mechanisms given that substrate protein degradation by a ubiquitin ligase is via the ubiquitin-proteasome pathway (Joanisse et al., 1998).

The identification of genes involved in ethanol-induced behaviours may facilitate a number of future experiments, including genetic screens towards identifying particular regions of the fly brain important for ethanol sensitivity (Rodan et al., 2002) and tolerance (Scholz et al., 2000). Accordingly, *hiw* and *hsp26* were manipulated in subsets of neurons in the fly's nervous system to identify the neuroanatomical loci that regulate their ethanol sensitivity and tolerance respectively.

Chapter 6.

Locus of Ethanol Sensitivity and Tolerance.

6 Locus of Ethanol Sensitivity and Tolerance

This chapter is a study to investigate the neuronal pathways underlying ethanol sensitivity of Hiw and tolerance of Hsp26. The work described in this chapter describes experiments to examine the neuroanatomical loci that modulate ethanol sensitivity and tolerance of these genes. The approach taken is to manipulate each gene in specific regions of the fly's brain using the GAL4/UAS targeted expression system. Section 6.1 provides a brief introduction to the study and describes the need for these experiments. Section 6.2 explains the experimental protocol and section 6.3 provides an analysis of the results. Finally, section 6.4 is a discussion of the investigation.

6.1 Introduction

Ethanol administration affects signaling in the nervous system and it has effects on the modulation of brain gene expression. Thus, alterations in neuronal structure, biochemistry and function have been considered the driving force behind the initiation of, and maintenance of drug addiction and dependence (Miguel-Hidalgo, 2009). For instance, neuroadaptive changes that occurred in the mesicorticolimbic system, a reward centre of the brain are thought to underlie the process of tolerance and dependence (Flatscher-Baden et al., 2006). Likewise, damage to the pre frontal cortex, PFC, the brain region responsible for many higher brain functions such as cognitive functions including planning ability have been reported in human alcoholics (reviewed in Flatcher-Bader, 2006). Certainly, this brain-oriented research has been very successful in providing very rich knowledge on the neuronal molecular pathways and brain circuits that are altered in response to drug effect and on how neuronal alterations modulate specific aberrant behaviours (Miguel-Hidalgo, 2009).

Thus, in order to understand the neuronal basis of ethanol sensitivity and tolerance, the characterisation of the pathways and the underlying brain regions mediating these phenotypes must be carried out. The requirement for the *hiw* and the *hsp26* genes to mediate ethanol sensitivity and tolerance respectively prompted us to inquire which regions of the fly brain are critical for Hiw and Hsp26 mediated ethanol sensitivity and tolerance respectively. We employed the GAL4/UAS

binary gene expression system (Brand and Perrimon, 1993) to target the expression of these genes in different brain regions. In *Drosophila*, this approach has been used to map neuroanatomical loci underlying behaviours such as ethanol sensitivity and tolerance (Urizar et al., 2007; Rodan et al., 2002), learning and memory (Connolly et al., 1996), courtship behaviour (O'Dell et al., 1995) and locomotion (Martin et al., 1999; Gatti et al., 2000). The availability of a collection of GAL4 lines with diverse expression patterns in the CNS (see http://www. Fly-trap.org; and Bloomington stock centres) enabled us to assess important regions including the ellipsoid body of the central complex, glial cells and centres involved in olfactory learning and memory.

The adult *Drosophila* brain (Figure 6.8 a) is made up of several principal neural centres including antennal and optic lobe and two central brain regions [i.e. the mushroom bodies (MBs) known to mediate classical olfactory conditioning (de Belle and Heisenberg, 1994), and the central complex (CC), involved in the higher–order brain functions including control of locomotion and visual pattern memory (Strauss and Heisenberg, 1993; Pan et al., 2009)]. The CC consists of four interconnected neuropilar bodies: the protocerebral bridge, the fan-shaped body, the paired noduli, and the ellipsoid body (Hanesch et al., 1989). The ellipsoid body neurons consist of large field neurons (i.e. R2/R4m neurons) that are critical for many brain functions including olfactory long-term memory consolidation (Wu et al., 2007), regulation of visual pattern memory (Pan et al., 2009) and regulation of ethanol sensitivity and tolerance (Urizar et al., 2007). The MB consists of networks of intrinsic neurons called Kenyon cells (KCs) derived from globuli cells located in the posterodorsal cortex above a prominent dendritic structure called the calyx, which receives olfactory information from the antennal lobes (ALs) via the inner antennocerebral tract (Tettamanti et al., 1997). During development the *Drosophila* MB neurons originate from four neuroblast per brain hemisphere, with each neuroblasts giving rise to an indistinguishable set of neurons and glia (Tettamanti et al., 1997). Glia constitute a support system for neuron; they provide high energy metabolic substrates to neurons to sustain neuronal activity and are responsible for recycling of the neurotransmitter glutamate at synapses (Freeman and Doherty, 2006).

The GAL4/UAS system was employed to individually drive expression of transgenes to overexpress, silence or rescue each gene activity in restricted brain regions. The flies' sensitivity or tolerance to ethanol was then measured.

6.2 Methods

The crosses were set up with P[GAL4] lines expressing *hiw* or *hsp26* gene in the whole nervous system of *Drosophila*. Additional GAL4 lines targeting a subset of neurons of the ellipsoid body, a neural structure that makes up part of the central complex (Renn et al., 1999), learning and memory centres of the *Drosophila* brain, mushroom bodies (MB) (Rodan et al., 2002), and the glial cells (Sepp et al., 2001) were chosen because drugs and ethanol were known to regulate these regions. Three separate crosses were set-up- one involving over-expression, the second involving functional knockdown using gene silencer RNA interference (RNAi) and the third rescue experiments.

6.3 Results

6.3.1 Neuronal expression of Hiw on ethanol sensitivity

We have previously shown that mutations affecting *hiw* gene in male flies results in a marked increase in ethanol sensitivity but no significant effect on ethanol tolerance. To determine whether functional knockdown of *hiw* using RNAi in the whole nervous system could mimic this behavioural phenotype, we used three independent hiw^{RNAi} transgene lines to silence *hiw* expression in all neurons using elav-GAL4, which expresses in all *Drosophila* neurons. As expected, pan neuronal silencing of *hiw* expression in all neurons leads to an increase in flies' ethanol sensitivity in both sedation and recovery assays (Figure 6.1 a and b). In contrast, we also wished to determine whether an increase in the endogenous levels of Hiw expression in all neurons could lead to resistance. This required that flies over-expressing Hiw proteins in the nervous system be assayed for ethanol sensitivity. Accordingly, flies carrying one wild type copy each of UAS-*hiw* transgene and elav-GAL4, and UAS-*hiw*ΔRING transgene and elav-GAL4 (Section 2.2, Table 2.1 in Materials and Methods) were tested for ethanol sensitivity in sedation and recovery

assays. The *hiw*ΔRING flies are identical to wild type *hiw* with the exception of two additional mutations in the first two cysteine residues (C4991 and C4994) in the RING finger domain. These residues have been shown to be required for *hiw* ubiquitin ligase function (Wu et al., 2005). Overexpression of Hiw in the nervous systems led to resistance to the sedating effects of ethanol and a shorter recovery time. Overexpression of HiwΔRING has no significant effect on ethanol sensitivity (Figures 6.1 c and d), indicating that the ubiquitin ligase function of Hiw mediates the ethanol response.

Next, to define specific brain regions and neural circuits in which *hiw* is required, GAL4 lines expressed in various discrete regions of the CNS under the control of endogenous enhancers, were used to express wild type *hiw* and *hiw*ΔRING (i.e. overexpression) or hiw^{RNAi} (i.e. functional knockdown) in a spatially restricted manner.

Flies carrying UAS-*hiw* and the individual P[GAL4] insertions with limited spatial expression in the brain were screened for sensitivity to ethanol in overexpression studies using the sedation and recovery assays. Of the 4 P[GAL4] lines tested, 2 displayed normal ethanol sensitivity in the presence of UAS-*hiw* or UAS-*hiw*ΔRING. These included lines with expression in the ellipsoid body (EB) and glial cells (Figures 6.1 c and d). In contrast, overexpression of wild type Hiw under the control of two P[GAL4] lines 201Y and OK107 led to a decrease in ethanol sensitivity (resistance) in the presence of UAS-*hiw* but not UAS-*hiw*ΔRING (Figures 6.1c and d). The lack of significant effect of UAS-*hiw*ΔRING confirms that the E3 ubiquitin ligase domain is required for the altered behaviour. Thus, overexpression of Hiw in a subset of CNS cells caused a specific reduction in ethanol sensitivity.

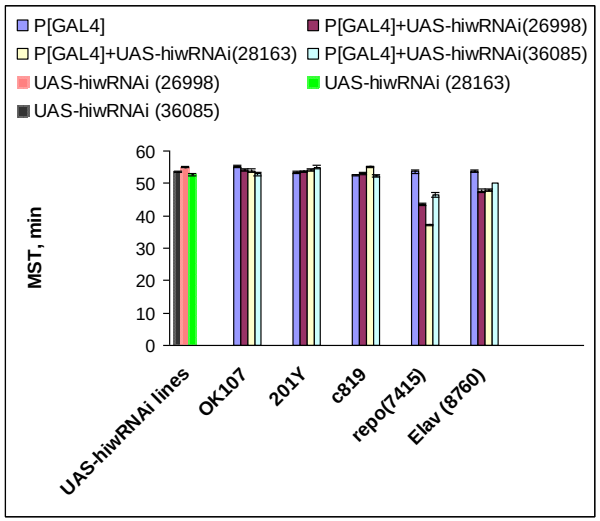
However, when hiw^{RNAi} gene silencer was employed to knockdown *hiw* gene expression in a spatially restricted manner, somewhat surprising results were obtained. Of the 4 P[GAL4] lines tested, 201Y and OK107 shown to cause decreased ethanol sensitivity in over-expression studies surprisingly displayed normal sensitivity to ethanol in the presence of UAS-hiw^{RNAi} (Figures 6.1 a and b).

These two lines drive expression in the Mushroom Bodies, which mediate learning and memory in the fly (Crittenden et al., 1998).

In contrast, expression of *hiw*^{RNAi} transgene in the presence of c819-GAL4 or repo-GAL4, which drive expression in the ellipsoid bodies or glia, respectively led to an increase in ethanol sensitivity (Figures 6.1 a and b). It should however, be noted that whereas driving the RNAi construct with repo-GAL4 caused enhanced ethanol sensitivity measured in both recovery and sedation assays, driving with c819-GAL4 displayed an increase in ethanol sensitivity only in the recovery assay. This raises the possibility that this region of the brain regulates the ability to recover from ethanol sedation but not susceptibility to ethanol sedation.

Figure 6.1. Transgenic expression of Hiw in specific brain regions alters ethanol sensitivity in the sedation and recovery assays. A, hiw^{RNAi} expression under the control of repo-GAL4 and elav-GAL4 resulted in decreased MST while that of OK107, 201Y and c819 showed no significant effects on MST. One-way ANOVA revealed a significant effect of genotype for the two P[GAL4] lines: repo and Elav ($P < 0.001$). B, The MRT of c819, repo and Elav was altered in the presence of UAS-hiw^{RNAi} transgenes. One-way ANOVA revealed a significant effect of genotype for the three P[GAL4] lines: c819, repo and Elav ($P < 0.001$). C and D, UAS-hiw overexpression under the control of OK107, 201Y and Elav resulted in increased MST and decreased MRT while that of c819 and repo showed no significant effects on both MST and MRT. One-way ANOVA revealed a significant effect of genotype for the three P[GAL4] lines: OK107, 201Y and Elav ($P < 0.0001$). In all figures, n=5 and error bars indicate SEM

a



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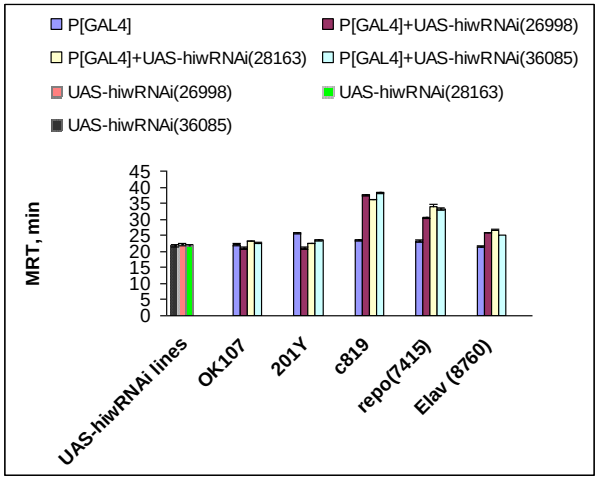

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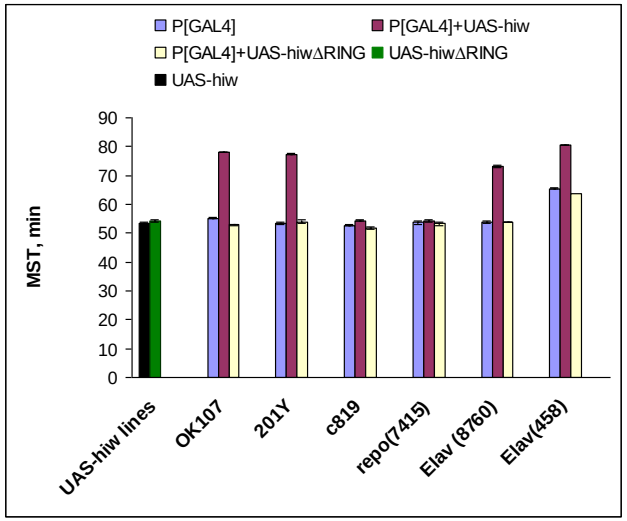

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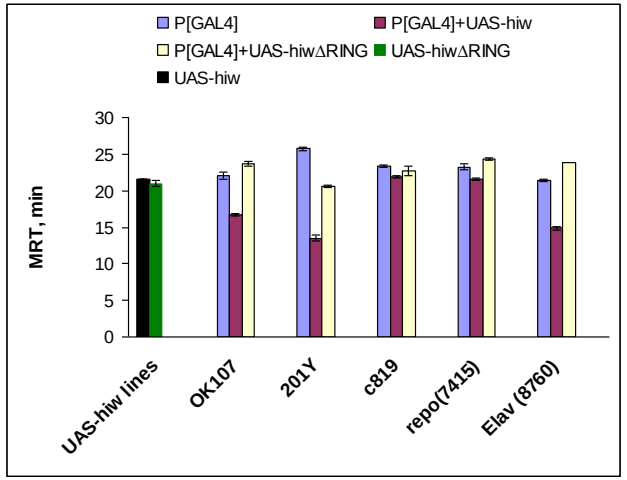


6.3.2 Effect of mushroom bodies in the regulation of Hiw ethanol sensitivity

Results obtained from over-expression studies are often difficult to interpret and must always be treated with caution. Nevertheless, the reduced ethanol sensitivity seen in the presence of UAS-*hiw* but not UAS-*hiw*ΔRING when wild-type Hiw is over-expressed in the mushroom bodies, suggests that this region of the brain regulates the ubiquitin-ligase mediated ethanol-sensitivity of *hiw*. Conversely, the fact that silencing of *hiw* expression in the mushroom bodies did not result in any significant alterations in ethanol response, indicate that while mushroom bodies may be playing a role in regulating ethanol sensitivity, Hiw is not involved. Thus, it is plausible that other ubiquitin ligases exist that probably mediate ethanol sensitivity in this region of the brain and these ligases buffer the cell from the sedating effects of ethanol and led to the reduced ethanol sensitivity phenotype seen in the overexpression studies.

6.3.3 Neuronal expression of Hsp26 on ethanol sedation (sensitivity)

We have previously shown that *hsp26* mutations do not affect the sensitivity of flies to ethanol in the sedation assay. To determine whether silencing of Hsp26 expression in the whole nervous system alters ethanol-induced behaviour, *hsp26*^{RNAi} transgene line was used to knockdown the expression of *hsp26* gene in all neurons using elav-GAL4, which expresses in all *Drosophila* neurons. As expected, pan neuronal silencing of *hsp26* expression in all neurons did not result in any significant alterations in ethanol response (Figure 6.2 a). In contrast, we also wished to determine whether an increase in the endogenous levels of Hsp26 expression in all neurons alters behavioural response to ethanol. Thus, flies carrying one wild type copy each of UAS-*hsp26* transgene and elav-GAL4 (Section 2.2, Table 2.1 in Materials and Methods) were tested for ethanol sensitivity in sedation assay. Overexpression of Hsp26 in the nervous systems surprisingly led to resistance to the sedating effects of ethanol (Figures 6.2 b), suggesting a role for Hsp26 in ethanol sensitivity.

Next, to determine whether expression of *hsp26* in specific brain regions alters ethanol-sensitivity in the sedation assay, GAL4 lines expressed in various discrete regions of the CNS under the control of endogenous enhancers, were used to express wild type *hsp26* (i.e. overexpression) or *hsp26*^{RNAi} (i.e. functional knockdown) in a spatially restricted manner.

Flies carrying UAS-*hsp26* and the individual P[GAL4] insertions with limited spatial expression in the brain were screened for sensitivity to ethanol in overexpression studies using the sedation assay. Of the 4 P[GAL4] lines tested, 2 displayed normal ethanol sensitivity in the presence of UAS-*hsp26*. These included lines with expression in the ellipsoid body (EB) and glial cells (Figure 6.2 b). In contrast, overexpression of wild type Hsp26 under the control of two P[GAL4] lines 201Y and OK107 led to a decrease in ethanol sensitivity (resistance) in the presence of UAS-*hsp26* (Figure 6.2 b).

However, when *hsp26*^{RNAi} gene silencer was employed to knockdown *hsp26* gene expression in a spatially restricted manner, all the 4 P[GAL4] lines tested, including the 201Y and OK107 shown to cause decreased ethanol sensitivity in over-expression studies, displayed normal sensitivity to ethanol in the presence of UAS-*hsp26*^{RNAi} (Figures 6.2 a).

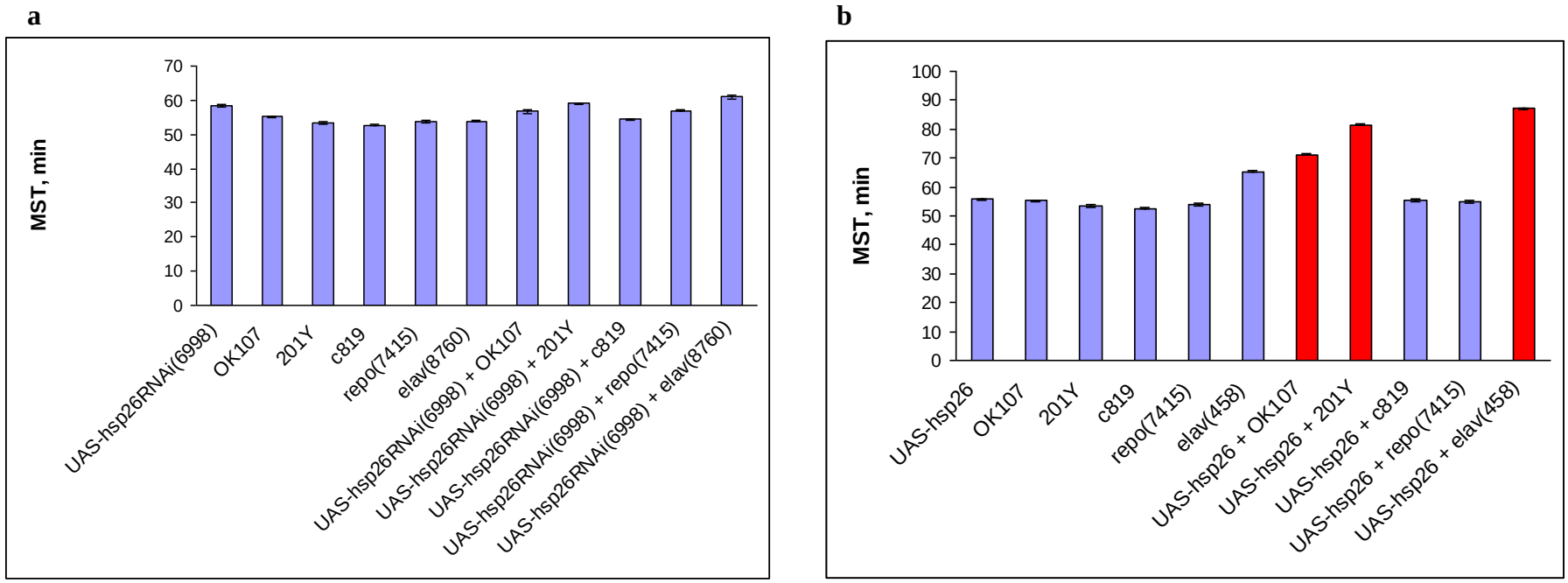


Figure 6.2. Overexpression of Hsp26 in the whole nervous system (elav (458)) and mushroom bodies (OK107 and 201Y) led to significant alterations in ethanol sensitivity but RNAi expression of *hsp26* in these structures showed no significant alterations in ethanol sensitivity. (a) Right panel shows the mean sedation time (MST) from the RNAi experiments of flies tested for ethanol sensitivity. No significant alterations in behaviour were seen in any of the *hsp26*^{RNAi} driven brain structures that were assayed. (b) Left panel shows that while increased Hsp26 expression in the presence of P[GAL4] lines OK107, 201Y and elav led to reduced ethanol sensitivity (red bars), expression in the c819-GAL4 and repo-GAL4 did not exhibit any significant alterations in ethanol response when compared with either the trangene alone or the corresponding GAL4 driver. The asterisk indicates significant differences as determined by a One-way ANOVA and post hoc Newman-Keuls (n= 5 experiments; $^{*}p < 0.01$). In all panels, error bars represent s.e.m.

6.3.4 Effect of mushroom bodies in the regulation of Hsp26 ethanol sensitivity

As discussed above, the effect of mushroom bodies on ethanol sensitivity appear to require increase in the endogenous levels of *hsp26* gene, because silencing this gene in these structures did not result in any significant alterations in ethanol sensitivity compared to the control flies. The overexpression behaviour of *hsp26* gene in the MBs correlates with the microarray data. When we feed flies ethanol, transcription of *hsp26* goes up. This heat shock response is immediate and induced in a protective way to buffer the cell from further ethanol insults and therefore leads to the delayed ethanol response of flies overexpressing this gene in the MBs. Consistent with this is the reduced ethanol sensitivity seen when *hsp26* gene is overexpressed in all neurons using a pan neuronal driver elav-GAL4 (Figure 6.2 b). Under physiological conditions, Hsps have been shown to acts as molecular chaperones that ensure cellular protein homeostasis and cell protection (Aït-Aïssa et al., 2003, Singh et al., 2009). The behaviour of *hsp26* in the MBs is specific because overexpression of this gene in the ellipsoid body neuron using the c819-GAL4 driver did not lead to any alterations ethanol response.

Conversely, the normal ethanol phenotypes seen when *hsp26* expression is silenced in the mushroom bodies (MBs) suggests two possible mechanisms: First, it is known that small heat shock proteins (sHsps) in *Drosophila* are clustered within the same locus on chromosome 3L (Joanisse et al.,1998) , it is therefore possible that silencing *hsp26* expression in the MBs did not result in overt response to ethanol sensitivity because other chaperones exists in these structures that compensate for the effect of *hsp26* and that this *hsp26* gene is redundant in the MBs. However, if *hsp26* mutates to lethality, this scenario is less likely to be true because removing this gene, the flies will less likely to have survived. Though, evidence from Flybase annotations (www.flybase.org) for *hsp26* gene revealed that most alleles of *hsp26* are not lethal and all the reported alleles insert in the 5' non-coding exon or regulatory region, we still can not rule out the possibility that the gene does mutate to lethality. The second possibility is that the RNAi technique might not be effective enough to suppress the *hsp26* gene function in the MBs. This stems from the observation that the RNAi transgene cannot be knocking down the gene properly because *hsp26*,

earlier shown to abolish ethanol tolerance from the mutant analysis, show reduced ethanol tolerance development when UAS-*hsp26*^{RNAi} expression is induced in the whole glia or nervous system.

6.3.5 Pan-neuronal expression rescues the *hsp26*^{EY10556} ethanol tolerance phenotype

It has been previously shown that flies carrying hsp26 mutations are unable to acquire tolerance to ethanol. *hsp26* mutations do not affect the sensitivity of flies to ethanol but they are unable to acquire tolerance. To determine whether silencing of Hsp26 expression in the nervous system can mimic the ethanol tolerance phenotypes observed in the *hsp26* mutant flies, we used the GAL4/UAS gene silencing system to silence *hsp26* expression in the nervous system of wild-type flies. We first used elav-GAL4, a pan neural driver to express the *hsp26*^{RNAi} transgene in all neurons. Flies carrying the elav-GAL4 driver and a P[UAS-*hsp26*^{RNAi}] were generated and tested for ethanol tolerance in the sedation assay. Pan neuronal silencing of *hsp26* gene results in reduced ethanol tolerance (Figure 6.3). Neither the elav-GAL4 driver nor the P [UAS-hsp26^{RNAi}] alone displayed reduced ethanol tolerance, indicating that the tolerance defect observed required the presence of both of these drivers. However, mutations in *hsp26* were previously shown to prevent the development of tolerance in flies. One interpretation of these results is that the reduced ethanol tolerance seen indicated that the silencing activity of *hsp26*^{RNAi} did not completely eliminate *hsp26* gene activity and the residual *hsp26* gene expression results in the reduced levels of tolerance seen. Consistent with this, is the observation that *hsp26*^{EY10556}/+ male flies displayed ethanol tolerance above the intermediate levels between homozygous *hsp26*^{EY10556} and wild-type control flies (Chapter 5, Figure 5.5).

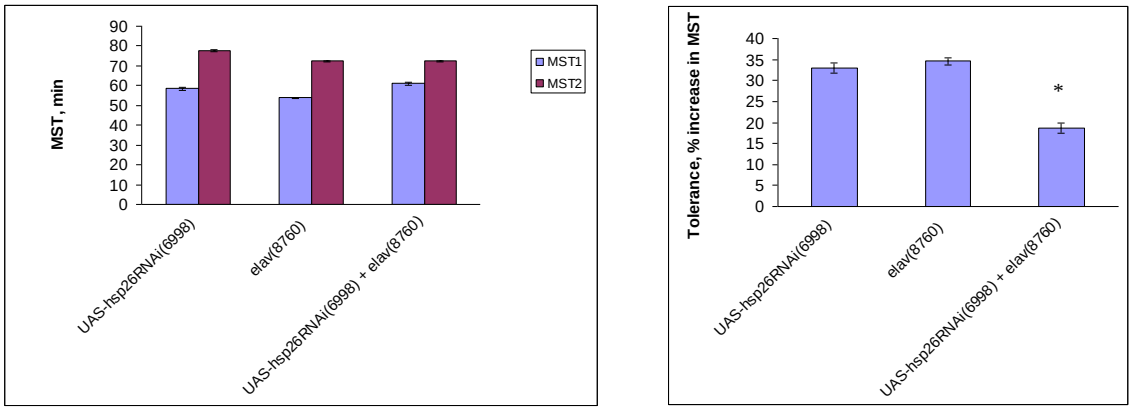


Figure 6.3 | Hsp26^{RNAi} expression in the nervous system reduced tolerance
Left panel shows the mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol tolerance (second exposure, purple bars). Right panel shows the development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. *hsp26*^{RNAi} expression when driven by 8760 (elav-GAL4) shows significantly reduced ethanol tolerance compared with either transgene alone. *$p < 0.001$; n=5 experiments. In all panels, error bars represent s.e.m.

To further confirm the role of the *hsp26* gene in the development of ethanol tolerance, the ability of a *hsp26* transgene to rescue the tolerance defect seen in *hsp26*^{EY10556} was examined. elav-GAL4/UAS-*hsp26*; *hsp26*^{EY10556} flies were tested for ethanol tolerance (Appendix A.5 for the scheme used in generating these flies). Pan neuronal expression of wild-type *hsp26* restored the ethanol tolerance of *hsp26*^{EY10556} flies to wild-type control levels (Figure 6.4). The MST of the wild-type flies was not significantly different from the MST of the elav-GAL4; *hsp26*^{EY1055}/UAS-*hsp26*; *hsp26*^{EY10556} flies. Neither the elav-GAL4 driver nor the P[UAS-*hsp26*] transgene alone altered the ethanol tolerance of the *hsp26*^{EY10556} mutant flies substantially (Figure 6.4). Taken together, both these experiments prove that abolition of *hsp26* activity is responsible for the *hsp26*^{EY10556} ethanol tolerance phenotype and that *hsp26* regulates rapid ethanol tolerance by acting within the nervous system.

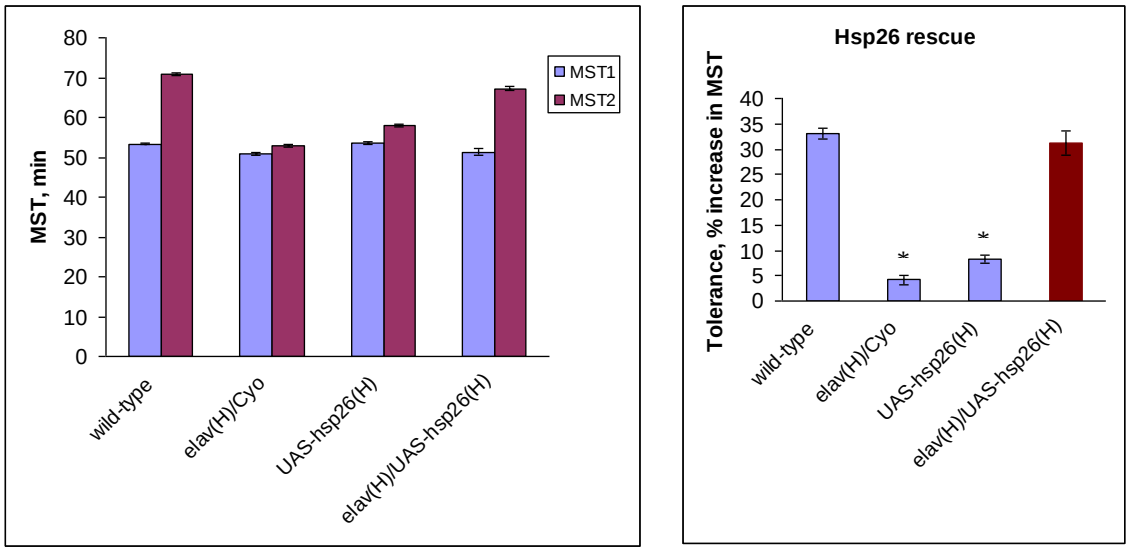


Figure 6.4| Hsp26 expression in the nervous system rescued rapid tolerance

The *hsp26*^{EY10556} tolerance defect can be rescued by expression of a UAS-hsp26 transgene in the nervous system under the control of elav-GAL4 driver. Mutant flies in an *hsp26*^{EY10556} background (denoted with H in figure) carrying either transgene alone show a marked defect in tolerance development similar to that of *hsp26*^{EY10556} mutant flies, whereas flies carrying both transgenes show normal tolerance. *p < 0.0001; n=5 experiments. In all panels, error bars represent s.e.m.

To determine whether increased expression of endogenous Hsp26 in the nervous system alters ethanol tolerance behaviour, elav-GAL4/UAS-*hsp26* flies, in a wild-type were also tested for rapid ethanol tolerance. The overexpression of *hsp26* in the nervous system did not alter rapid ethanol tolerance (Figure 6.5) when compared to elav-GAL4 driver or the P[UAS-hsp26] transgene alone. This suggests that ethanol tolerance was not affected by raised Hsp26 levels in the nervous system.

Next, to define specific brain regions in which Hsp26 regulates ethanol tolerance, UAS-hsp26^{RNAi} (see below) and UAS-hsp26 were driven by GAL4 lines that direct expression in discrete regions of the nervous system. All of the 4 P[GAL4] lines tested in the overexpression studies, repo-GAL4 (7415; glia), c819-GAL4 (ellipsoid bodies), 201Y-GAL4 and OK107-GAL4 (Mushroom Bodies) displayed normal ethanol tolerance in the presence of UAS-hsp26 (Figure 6.5).

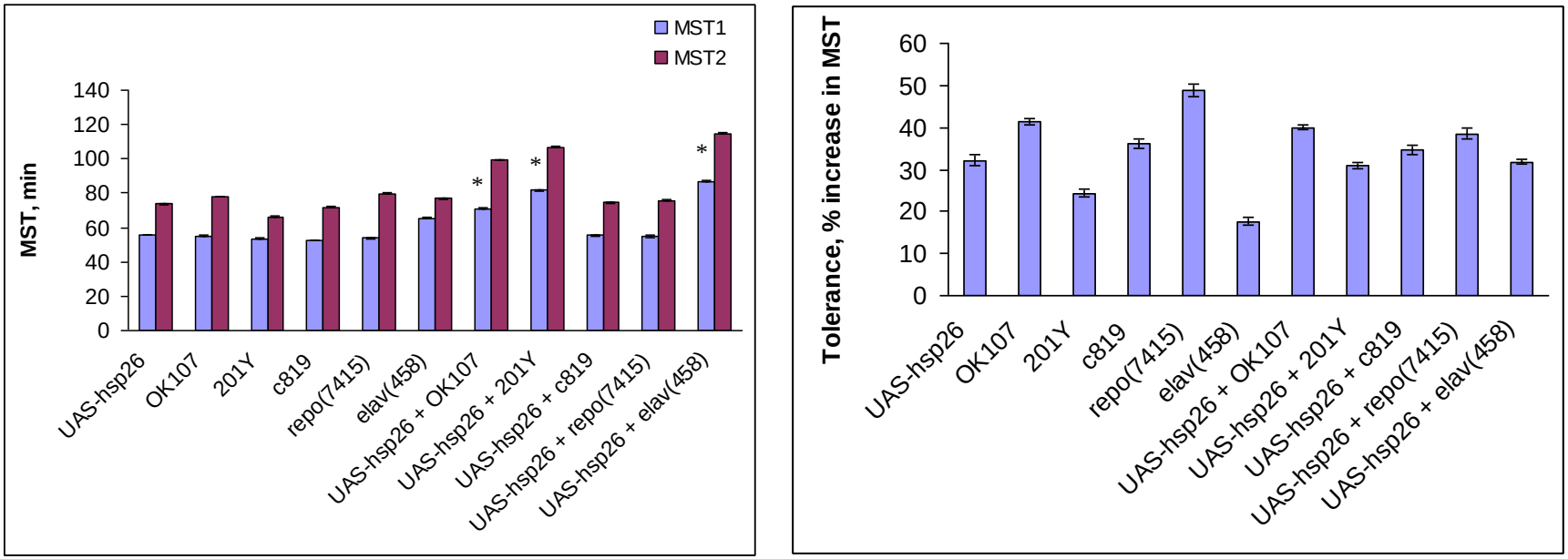


Figure 6.5. Overexpression of Hsp26 in the whole nervous system (elav (458)) and mushroom bodies (OK107 and 201Y) led to significant alterations in ethanol sensitivity but no significant effect on tolerance. Left panel shows the mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol tolerance (second exposure, purple bars). Right panel shows development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. Hsp26 overexpression in the whole nervous system and the mushroom bodies show significantly reduced sensitivity (increase in MST) but no effect on tolerance compared with either UAS-hsp26 transgene alone or corresponding GAL-4 driver ($P < 0.001$). However, increase Hsp26 expression in the presence of c819-GAL4 and repo-GAL4 did not exhibit any significant alterations in ethanol response when compared with either the trangene alone or the corresponding GAL4 driver. The asterisk indicates significant differences as determined by a One-way ANOVA and post hoc Newman-Keuls (n= 5 experiments; $^{*}p < 0.01$). In all panels, error bars represent s.e.m.

However, when *hsp26*^{RNAi} gene silencer was used to knockdown *hsp26* gene expression in a spatially restricted manner using the four GAL4 enhancer trap lines that direct expression in discrete regions of the nervous system, 3 of the P[GAL4] lines displayed indistinguishable ethanol tolerance when compared to their respective P[GAL4] line or UAS-hsp26^{RNAi} transgene alone. These included lines with expression in the mushroom bodies (201Y and OK107), and the ellipsoid body of the central complex (c819) (Figure 6.6), suggesting that both ellipsoid body and mushroom bodies regions of the brain do not regulate *hsp26* ethanol tolerance phenotype. The non-significant alterations in ethanol tolerance seen when *hsp26* expression was silenced in the MBs and the observation that this region of the brain possibly regulate Hsp26 ethanol sensitivity from the overexpression studies earlier described, prompted us to perform a proper rescue experiment to confirm whether this region of the brain does not regulate the Hsp26 ethanol tolerance phenotype. 201Y-GAL4/UAS-*hsp26*; *hsp26*^{EY10556} flies were tested for ethanol tolerance (see Appendix A.5 for the scheme used in generating these flies). Expression of wild-type *hsp26* in the MBs did not restore the ethanol tolerance phenotype of *hsp26*^{EY10556} flies to wild-type control levels (Figure 6.7). Taken together, both of these experiments prove that mushroom bodies are not involved in the regulation of *hsp26*^{EY10556} ethanol tolerance phenotype.

In contrast, expression of *hsp26*^{RNAi} transgene in the presence of repo-GAL4, which drives expression in the glia, led to a significantly reduced rapid ethanol tolerance phenotype compared to repo-GAL4 driver or UAS-*hsp26*^{RNAi} transgene alone (Figure 6.6). This raises the possibility that the glia specific region of the brain regulates the ethanol tolerance defects.

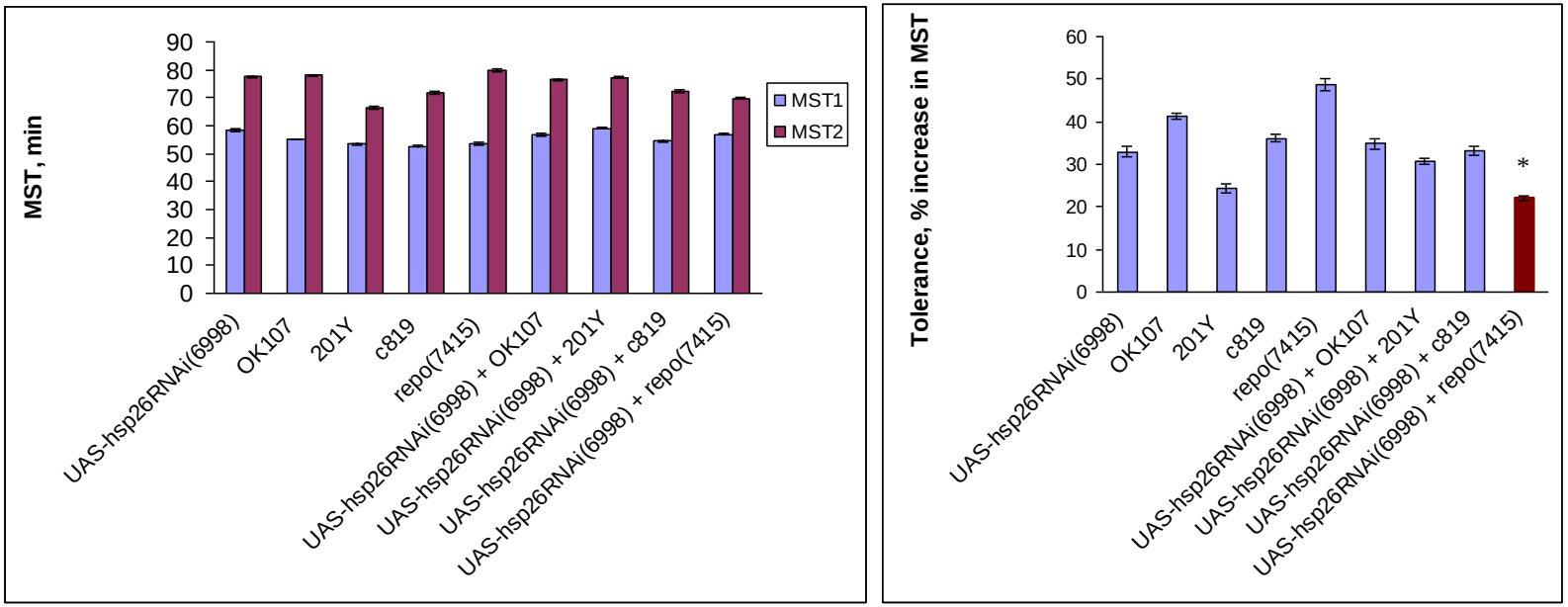


Figure 6.6. Spatially restricted functional knockdown of Hsp26 in the brain with repo (7415) led to reduced ethanol tolerance. Left panel shows the mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol tolerance (second exposure, purple bars). Right panel shows development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. *Hsp26*^{RNAi} expression in repo (7415) show significantly reduced tolerance compared to UAS-hsp26^{RNAi} transgene alone or repo-GAL4 driver ($P < 0.0001$). However, *Hsp26*^{RNAi} expression in the presence of OK107, 201Y and c819 did not exhibit any significant alterations in ethanol tolerance when compared either the trangene alone or the corresponding GAL4 driver. The asterisk indicates significant differences as determined by a One-way ANOVA and post hoc Newman-Keuls (n= 5 experiments; $^{*}p < 0.01$). In all panels, error bars represent s.e.m.

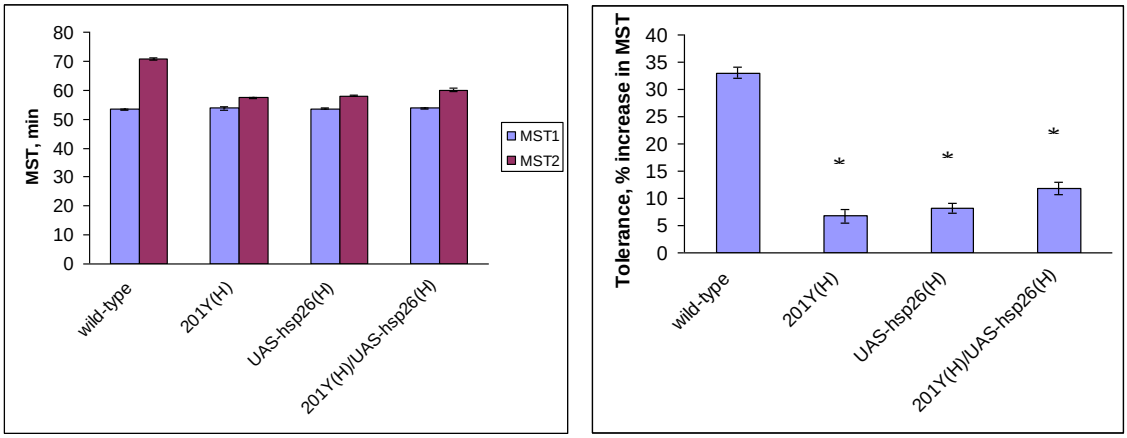
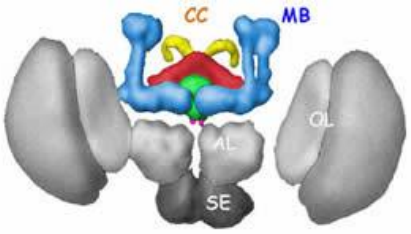


Figure 6.7| Hsp26 expression in the MBs did not rescue rapid tolerance

The *hsp26*^{EY10556} tolerance defect cannot be rescued by expression of a UAS-hsp26 transgene in the mushroom bodies under the control of 201Y-GAL4 driver. Mutant flies in an *hsp26*^{EY10556} background (denoted with H in figure) carrying either transgene alone show a marked defect in tolerance development similar to that of *hsp26*^{EY10556} mutant flies. In addition, flies carrying both transgenes also display similar tolerance defect. *p < 0.0001; n=5 experiments. In all panels, error bars represent s.e.m.

http://homes.bio.psu.edu/



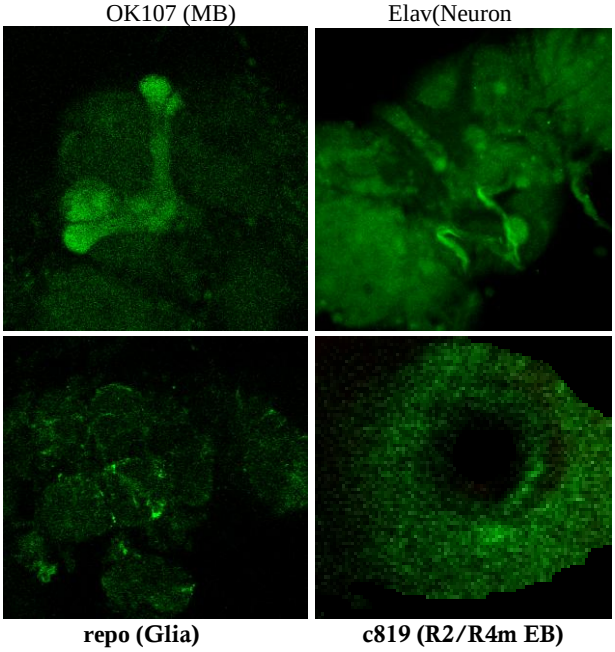


Figure 6.8. (a), Cartoon of the *Drosophila* brain structures; blue, mushroom bodies (MB); red, fan shaped bodies; green, ellipsoid bodies; pink, noduli; yellow, proto-cerebral bridge. CC, central com-plex that includes aforementioned. four structures., AL, antennal lobes; SE, subesophageal ganglia; OL, optic lobes. Image taken from http://homes.bio.psu.edu/people/faculty/han/ResearchInterests.htm.

(b), Confocal images of the adult *Drosophila* brain showing P[GAL4] directed expression of GFP in the MBs (OK107; 854), neurons (Elav; 8765), glia cells (repo; 7415) and the R2/R4m ring neurons of EB (c819) are shown.

6.4 Chapter Discussion

The mechanisms governing ethanol actions are only partially understood and as such required detailed characterisation of the neuroanatomical loci involved in mediating different ethanol phenotypes such as sensitivity and tolerance. Thus, characterising the brain structures involved in the modulation of *hiw* ethanol sensitivity and *hsp26* ethanol tolerance in *Drosophila* is vital to identify the neural circuits regulating these behaviours. Here, it was shown that by individually manipulating these genes in the flies' brain distinct brain regions can be linked to ethanol sensitivity and tolerance to ethanol. Using two behavioural assays that measures two distinct aspects of ethanol response (i.e. sedation to and recovery from ethanol intoxication), we show that Hiw signaling in the ellipsoid body neurons regulates distinct aspects of the behavioural response to ethanol and in this case recovery from ethanol sedation. In addition, it was shown that the ubiquitin ligase function of *hiw* is required to reduce the flies' sensitivity to the effects of ethanol on sedation and recovery.

The previous section has shown that, the glial cells, the ellipsoid body neurons of the central complex (CC) and the mushroom bodies (MBs) may be involved in developing sensitivity and tolerance to ethanol. A role for these three structures in the modulation of ethanol-induced behaviours has been previously reported. For instance, a role for glia in the responses to drugs of abuse in both fly and mammals has been extensively reviewed (Bainton et al., 2005, Haydon et al., 2009). Fly glia are known to be involved in the modulation of neurotransmission and behaviour (Jackson and Haydon, 2008). Mutants with structural brain abnormalities located in the MBs and the CC show significantly reduced ability to develop tolerance (Scholz et al., 2000). This same study by Scholz et al., (2000) has shown that two P[GAL4] lines with expression in a subset of CC neurons, the small field neurons that connects the ellipsoid body (EB) with other CC structures, display significantly reduced tolerance in the presence of a tetanus toxin transgene.

It has been previously documented that the activation of the heat shock pathway cascade by moderate levels of alcohol promotes neural survival (Pignataro et al., 2007). Several studies indicate that HSPs expressed in glial cells principally belong

to the group of small-molecular-weight HSPs including Hsp26, which could play an important role in the metabolic changes undergoing the glia response to neural damage (Acarin et al., 2002). Interestingly, this study has shown that silencing of *hsp26* gene in the glia reduced tolerance to ethanol while the ethanol tolerance defect seen in *hsp26* mutant flies can be rescued by pan-neuronal expression of wild-type *hsp26*. This raises the possibility that Hsp26 may be acting through a mechanism to protect the neural cells including the glia from ethanol-induced damage, thus maintaining the integrity of the glia and promoting neural cell stabilisation and survival. In the glial cells, Hsp26 may also function in cooperation with ubiquitin-proteasome system (UPS) through protein refolding and targeting of irreversibly damaged proteins for degradation in order to prevent proteotoxic damage (Joanisse et al., 1998).

Exposure of both mammals and flies to varying concentrations of ethanol has been reported to have distinct behavioural consequences (Rodan et al., 2002). In *Drosophila*, these can be separated using different assays such as sedation and recovery assays (this Study), inebriometer and locomotor tracking system (Rodan et al., 2002). Using sedation and recovery assays, I have shown that RNAi mediated functional knockdown of *hiw* under the control of c819 (as discussed above), which did not affect sensitivity to the sedative effects of ethanol in the sedation assay, altered sensitivity to the recovery from ethanol sedative effect. Thus, spatially refined expression of hiw^{RNAi} transgene to conditionally decrease the concentration of Hiw amounts in different sets of brain neurons cells, affects distinct aspects of acute behavioural effects of ethanol.

The R2/R4m neurons in the ellipsoid body have recently been shown to be involved in ethanol sensitivity and tolerance (Urizar et al., 2007). The same structures have later been implicated in NMDA-receptor dependent olfactory long-term memory consolidation (Wu et al., 2007) and in visual pattern memory (Pan et al., 2009). This study also implicates the EB in ethanol sensitivity and suggests that *hiw* plays a role at least in this tissue. Though, the exact role of the ellipsoid body neurons in Hiw mediated ethanol sensitivity is not known, we can infer from previous studies on the larval NMJ (Wan et al., 2000, Wu et al., 2005) that Hiw

(through its functional RING finger domain) may mediate synaptic signalling in these neurons. Thus, Hiw-dependent regulation of synaptic morphology may be lost in flies with reduced levels of *hiw* in these neurons and this loss leads to an increase in ethanol sensitivity measured in the recovery assay. In addition, the fact that olfactory learning and ethanol sensitivity appears to share similar molecular mechanism (discussed below) may indicate a role for Hiw in some forms of memory consolidation. With the RNAi result that indicated a necessary role of EB neurons, it could only be suggested at this time that the ellipsoid body neurons may be a brain region where Hiw functions to affect ethanol sensitivity measured in the recovery assay. Nevertheless, further investigation is required to confirm the role of this brain region in *hiw*-mediated ethanol sensitivity.

The Hiw E3 ubiquitin domain is a RING finger domain essential for *hiw* function (Wu et al., 2005). In yeast, a role for ubiquitin in ethanol sensitivity has been reported. Two yeast mutant strains- yeast ubiquitin ligase, Rsp5 and ubiquitin-conjugating enzyme, Ubc4 have been implicated in increased ethanol sensitivity (Hirashi et al., 2009). This evolutionary conservation of function between *Drosophila* and yeast ubiquitin proteins confirms the importance of Hiw-mediated protein ubiquitination in the regulation of ethanol-induced behaviours.

Protein ubiquitination has been reported to be required for many cellular processes involving protein degradation such as cell-cycle (Hershko, 1997), endocytosis (Staub and Rotin, 2006) and the stress response (Wolf and Hilt, 2004) while mutations in proteins in the ubquitin-proteasome system (UPS) lead to ataxia in mice (Wilson et al., 2002). In *Drosophila*, protein degradation via the UPS is an important negative regulatory mechanism of NMJ growth, as revealed by striking overgrowth in *hiw* mutants (Wan et al., 2000). The mutation of the first two cysteine residues (C4991 and C4994) in the RING finger domain in *hiw* abolishes *hiw* ubiquitin ligase function and is shown to decrease sensitivity to ethanol in the MBs. This raises the possibility that *hiw* functions in cooperation with an ubiquitin ligase in the presence of ethanol and may be involved in the ubiquitination of ethanol induced denatured proteins via the UPS. Of particular interest is the role of the E3 ubiquitin ligase domain of Hiw in kinase signaling pathway. Studies have

shown that Hiw E3 ubiquitin ligase restricts synaptic growth primarily by downregulation of Wallenda, (Wnd), a MAP kinase kinase (Collins et al., 2006). It is likely that this domain plays an important role in the neuroadaptation underlying ethanol-induced behaviour by downregulation of Wnd because *highwire; wallenda* double mutant ($hiw^{ND8};wnd^{1}/wnd^{2}$) have been shown to completely suppress the *highwire* synaptic overgrowth phenotype (Collins et al., 2006). Wallenda has been shown to be essential for synaptic overgrowth caused by both overexpression of a ubiquitin hydrolase and a loss of a ubiquitin ligase and the gene is reported to behave like a candidate substrate for ubiquitination that could mediate synaptic overgrowth (Collins et al., 2006), suggesting a model that *wallenda* is denatured and degraded in the presence of ethanol and that the ubiquitin ligase function in *hiw* is involved in the specific degradation of abnormal *wallenda* proteins. A prediction of this model is that *highwire; wallenda* double mutant ($hiw^{ND8};wnd^{1}/wnd^{2}$) will exhibit normal ethanol sensitivity.

The mechanisms regulating olfactory learning and ethanol sensitivity appear to share similar molecular components as several olfactory learning mutants such as *amnesiac*, *rutabaga*, and the cell adhesion molecule *fasciclin II*, showed altered ethanol sensitivity (Moore et al., 1998, Cheng et al., 2001). These mutants have been reported to be expressed in the fly's MBs [(see Roman and Davis, 2001, for *rutabaga* and *fasciclinII*) and (Waddell et al., 2000 for *amnesiac*)]. The MBs also play important roles in sleep homeostasis that is modulated via the cAMP/PKA pathway (Joiner et al., 2006). It is therefore, interesting that the two MB drivers used in our studies may be playing a role in the regulation of sensitivity to the sedative effects of ethanol in both Hiw and Hsp26 overexpression studies.

In addition, the cAMP-dependent protein kinase (PKA) signaling in one of the two MBs line used in this study, 201Y has earlier been shown to regulate sensitivity to the effects of ethanol on both motor coordination (measured in the inebriometer) and sedation (measured in the locomotor tracking assay) (Rodan et al., 2002). However, the altered ethanol sensitivity seen in this line was shown to be due to few neurons outside of the MBs expressing GAL4 in 201Y as chemical ablation of the MB did not alter ethanol sensitivity measured in the inebriometer (Rodan et al.,

2002). This same study also showed that the expression of transgene that inhibit PKA activity (i.e. PKA^{inh}) under the control of a P[GAL4] line with expression in the MB neurons did not alter flies ethanol sensitivity (Rodan et al., 2002). Nevertheless, the two independent GAL4 lines-201Y and OK107, that drive expression in the MBs in our study have in common the ß and γ lobe neurons of the MBs and may possibly suggest a role for these neurons in the regulation of ethanol sensitivity.

Finally, the fact that these two lines failed to regulate sensitivity to ethanol in the RNAi reciprocal rescue experiments suggests that such pleiotropic behaviour of these genes in the MBs in both overexpression and RNAi expression could be investigated further using genetic tools. For example, mutations affecting mushroom body development (e.g. mushroom body miniature, *mbm* or mushroom body deranged, *mbd*) could be assayed for ethanol sensitivities in our sedation paradigm. Screening with systematic deficiency sets having molecular defined break points could also be carried out to identify MB genes involved in ethanol sensitivity. To determine whether overexpression of these genes in MBs contributes to the reduced sensitivity of 201Y+UAS-hsp26, 201Y+UAS-hiw, OK107+UAS-hsp26, and OK107+UAS-hiw flies, we could treat larvae of these genotypes with hydroxyurea- a chemical previously demonstrated to have the ability to selectively ablate the MBs (de Belle and Heisenberg, 1994, Rodan et al., 2002), and determine their sensitivity to ethanol after MBs ablation using our sedation assay protocol. This protocol has been proven to be effective at unravelling the real effect of MBs on ethanol sensitivity (Rodan et al., 2002).

Chapter 7.

Summary and Further Work.

7 Summary and Further Work

7.1 Introduction

In this thesis, computational methods were used to identify differential patterns of gene expression on the 2 h and the time-course microarray data. Most of the differentially expressed genes identified encode several different categories of proteins, some of which are involved in signaling, stress and ubiquitination pathways. Results from these two analyses have shown that acute ethanol exposure elicits profound and multiple changes in many biological processes.

Seven of the genes from the 2 h data were linked to some changes in ethanol-induced behaviours. Further work on two of these genes revealed brain loci that may likely regulate these behavioural phenotypes. Thus, research carried out for this thesis shows that it has been possible to identify genes underlying alcohol-induced behaviours using *Drosophila* as a model by combining computational analysis with classical behavioural genetics. Genes with novel roles in regulating the behavioural responses to ethanol have been uncovered. Some of these may possibly influence alcohol dependence and thus, open up the possibilities for further analysis of the molecular mechanisms contributing to the disease.

In this final chapter, a summary is made of the findings from the successful statistical and behavioural analyses of the raw microarray data which led to the construction of the list of genes validated using behavioural and genetics methodologies. Finally, further possible directions for future work are considered.

7.2 Bioinformatics and behavioural genetics

The RMA and GCRMA algorithms in LIMMA statistical package have been used to derive a list of differentially expressed genes in response to an acute dose of ethanol in the 2 h microarray data. This work has shown that this approach was successful at identifying genes with altered transcription after ethanol exposure. 7 of these genes were confirmed by biological validation using sedation and recovery assays to be indeed implicated in acute ethanol response in *Drosophila*. 4 of the validated genes were later selected and implicated in some changes in ethanol

tolerance. As well as these, several genes, such as *Men*, *Cher*, and *Tsp42Ee* have also been shown by others to be implicated in ethanol response (Table 7.1).

Table 7.1. Some identified genes implicated in ethanol response

Gene	Function	sensitivity/ tolerance	role in alcohol ref(s).
Men	Malate metabolism; tricarboxylic acid cycle	S	Morozova et al., 2006
Cher	Cytoskeleton organisation and biogenesis	S	Berger et al., 2008
Rho	Nervous system development	S	Berger et al., 2008
Tsp42Ee	Toll signaling pathway	S	Berger et al., 2008
CG9086	Protein ubiquitination	R	Morozova et al., 2006
Hsp83[*]	Stress response; circadian rhythm	S, R	This study
mbf1[*]	Transcriptional regulation; dendrite morphogenesis	S, R	This study
ana[*]	Negative regulation of neuroblast proliferation	S	This study
hop[*]	Immune response; cell division	S	This study
Hsp26[*]	Aging; protein folding	R	This study
hiw[*]	BMP signaling pathway	S	This study
Axn[*]	Wnt receptor signaling pathway	S	This study

Of the genes shown to be significantly regulated in response to ethanol, 7 were validated in this study while 5 others have been reported in other studies. Ethanol sensitivity and tolerance were quantified in the sedation assay (this study) or inebriometer (Morozova et al., 2006 and Berger et al., 2008). S, increased or decreased ethanol sensitivity; R, increased or decreased ethanol tolerance. [*]Experimentally confirmed in this study.

It is of considerable interest that all the seven genes selected from the microarray list of differentially expressed genes were subsequently shown to affect the fly's behavioural response to ethanol, and it is especially noteworthy that these genes are involved in stress response (*hsp26*, *hsp83*, *hiw* and *mbf1*), signaling (*hop*, *axn*) and neurogenesis (*ana*). Interestingly, evidence for genes involved in each of these biological processes in regulating the effect of alcohol in higher organisms has been documented (Flatscher-Bader et al., 2006), but the mechanism underlying their ethanol-induced behavioural responses are not well understood. This study thus provides the basis for dissecting the roles of these biological processes in *Drosophila*, a relatively simple model genetic system.

Oxidative stress is one of the probable mechanisms involved in ethanol-induced neural damage (Bjork et al., 2006). Stress being an energy demanding process, requires that flies mobilise energy substrate to cope metabolically with the ethanol challenge. *Drosophila* cells thus undergo a series of biochemical and physiological changes in an attempt to cope with ethanol stress following exposure. Thus the increased transcript levels of *hsp26*, *hsp83*, *hiw* and *mbf1*, *axn* and *hop* genes, and a reduction in transcription of *ana* gene, all implicated in behavioural response to ethanol, may reflect a form of defense mechanism required to enhance the survival of the stressed cells. It seems plausible that acute ethanol exposure in flies is accompanied by a redox imbalance that triggers the coordinated upregulation of genes encoding stress (e.g. *mbf1*, *hiw*, *hsp26* and *hsp83*) and signaling (e.g. *hop*, *axn*) pathways and concurrent downregulation of gene (e.g. *ana*) involved in neuronal differentiation and growth. In addition, the implication of the activated signaling pathway in ethanol response suggests that genes encoding this pathway may mediate longer-term changes in gene expression while the downregulation of the neuronal gene (e.g. *ana*) may contribute to the compromise of neuronal functions due to ethanol exposure in the *Drosophila* head.

Analysis of the time-course microarray data showed that changes in expression of most genes peaked at 2 h. Here, the major gene expression changes is the stress response with the stress proteins and detoxification enzymes showing the most altered regulation with a peak of activity at 2 h which has declined by 4 h.

Interestingly, several transcription factors (e.g. *Sox14*, *tara*, *cbt* and *elb*) showed an increase in transcription that peaked at about 0.5 to 1 h and thus raises the possibility that proteins encoding these genes may be responsible for later transcriptional activation of other regulated genes.

Functional clusters of the differentially expressed genes from the 2 h data showed that most of the up-regulated genes seem to do so in response to changes in environment. The analysis also identified groups of upregulated genes that were involved in signaling pathways. Thus, these genes may regulate longer-term changes /processes (e.g. memory and learning, and synaptic plasticity). Most of the downregulated genes fall into the GO categories of metabolism. Many other regulated genes encode different categories of proteins involved in transcriptional regulation, protein ubiquitination, neurogenesis, and RNA-binding proteins and suggest that ethanol exposure produces a broad and coordinated response in gene expression.

Because the experimental design depends on reliable signal comparisons between non-ethanol control and ethanol-treated samples, expression values were calculated using moderated t-statistics by fitting a linear modelling contrast for these two conditions and adjusting the resultant raw p values by the false discovery rate method of BH (Bejamini and Hoschberg, 1995). Notably, despite the fact that a large number of 'ethanol-affected genes' are successfully identified by the microarray screening, some of the genes known to be affected by ethanol, such as *GABA-B*, *NMDA* receptors, *Fas2*, *rut*, *npf*, *hang*, *Tbh*, *amn*, *Slo*, and *jwa* were excluded by the selection criteria used in this work. These genes may likely constitute transcripts with low abundance in the adult *Drosophila* head that were missed by our detection analysis (e.g. *GABA-B-R3*, p = 0.11 and *Nmda1*, p = 0.12 which had absent calls). Indeed, some of the genes may be present at high levels but in a limited number of cells while others may be expressed at low levels in all the cells. In addition, about 6.8% (949 of the 14010) genes on the Affymetrix *Drosophila* Genome 1 Array chip are missing and cannot be included in any of the analyses carried out for this thesis. More importantly, the implication of some of these genes in alcohol response may be due to post-transcriptional and translational events as

they were not differentially expressed. Nevertheless, the sensitivity power of the two different algorithms employed coupled with the stringent FDR approach adopted in the analysis have helped to uncover a network of genes with altered transcription upon alcohol exposure with very few (if any) false positives.

By microarray and behavioural analyses, it was shown that transcription of the stress proteins *hsp26*, *hsp83* and the transcriptional regulator *mbf1* is altered after exposure to ethanol, and that this is coincident with a gain of tolerance. A hypothesis to explain the role of these proteins in tolerance would be that they play a protective role in the nervous system, and they are upregulated following ethanol sedation. This upregulation occurred to ensure cellular protein homeostasis and cell protection from ethanol insults. These genes are upregulated with a peak of transcription at 2 h after ethanol sedation in wild-type flies, a period which flies are known to display highest rapid tolerance (see section 5.3.1, Figure 5.2 of Chapter 5). It is therefore interesting that flies carrying mutations for these genes are unable to acquire normal tolerance and flies overexpressing *hsp26* display resistance on first exposure to ethanol.

Sensitivity to ethanol is a neuronal phenotype that is mediated by the *highwire* ubiquitin-ligase domain in flies. *Hiw* is upregulated between 0.5 and 2 h and downregulated between 3 and 4 h after ethanol sedation in flies' head in the microarray time course data (data not shown). Using the behavioural assay, it was demonstrated that *hiw* loss-of function mutation showed no effect on ethanol tolerance in male flies. Why this gene contributes to ethanol sensitivity and not tolerance to ethanol remains unclear. What it does show is that ethanol sensitivity and ethanol tolerance can be separated genetically, and that some genes can affect one type of ethanol induced behaviour but not another. It is, however, difficult to formulate a hypothesis about the behavioural role of the genes obtained from the time course microarray data. This is because the changes in expression of some of these genes could constitute many alternative events or changes taking place in different brain regions and/or at different times, some of which may be relevant to some ethanol-induced behaviours and not others.

The *Drosophila* central complex consists of four interconnected neuropilar bodies: the protocerebral bridge, the fan-shaped body, the paired noduli, and the ellipsoid body (Hanesch et al., 1989). The ellipsoid body neurons are critical structures of the fly central complex important for many brain functions including olfactory long-term memory consolidation (Wu et al., 2007), regulation of visual pattern memory (Pan et al., 2009) and regulation of ethanol sensitivity and tolerance (Urizar et al., 2007). Silencing the expression of *highwire* in the ellipsoid body using the GAL4/UAS systems leads to increased sensitivity only in the recovery assay. This suggests that this region of the brain may be regulating the ability to recover from but not susceptibility to ethanol sedation. There are P[GAL4] lines which preferentially express GAL4 in specific neurons of the ellipsoid bodies that are implicated in different functions. This would help elucidate the precise role of *highwire* regulating ellipsoid body function in mediating ethanol sensitivity.

This study has also implicated glia and mushroom bodies as likely mediators of the acute ethanol response in *Drosophila*. Silencing *hiw* expression in the glial cells causes enhanced ethanol sensitivity in both sedation and recovery assays. This same region of the brain was implicated in *hsp26* reduced rapid ethanol tolerance when driven with the $hsp26^{RNAi}$ construct. Finally, over-expression of *hiw* and *hsp26* in the mushroom bodies led to reduced ethanol sensitivity.

7.3 Possible directions for future work

Other statistical packages exist for analysing these microarray data. One of these is the significance analysis of microarrays (SAM) developed by Tusher et al. (2001) specifically to address the problem of FDR in microarrays. Here, they used the FDR approach to estimate non-significant genes by analysing permutations of the measurements. SAM also allows filtering of genes that are above or below a user defined n-fold change (delta value). This is essentially important because of the constant danger in microarray analysis of not including genes that are minimally altered but highly relevant (Nambiar et al., 2005). This method, though not used in this thesis, is expected to give similar results to that obtained from LIMMA.

Nevertheless, future investigators will be able to broaden the scope of some findings presented here. A follow-up study on the time course clustered data or using a new clustering tool to cluster gene lists produced based on their expression profiles in time series will provide a very interesting piece of work. For instance, the following questions could be addressed from the microarray time course clustering analysis:

1) Determine what classes of genes show immediate early and late responses respectively and reason whether these behaviour could have meaningful biological interpretations in relation to ethanol response.
2) Determine through cluster analysis whether genes showing time-dependent differential behaviour and whose levels are different from base line at the end of 4 h are implicated in ethanol tolerance in *Drosophila* when tested experimentally.
3) Determine through experimental validation whether an assumption that genes with similar gene expression patterns and following similar time course show similar response to ethanol sensitivity and/ tolerance and.,
4) Finally, determine whether the classes of genes in each cluster are governed by the same regulatory elements or transcriptional factors binding sites. This could be done by analysing the promoter sequences of early responsive genes to identify the conserved motifs.

Validation of the above hypotheses will offer new directions for more behavioural work in conjunction with microarray data. This may offer novel insights into the time-dependent regulation of gene expression monitoring under acute ethanol administration.

This research has also raised an important question on sex-specific differences in the fly's sensitivity and tolerance to an acute dose of ethanol. Thus, detailed research into this area may be helpful in elucidating the mechanisms governing this ethanol-induced differential behaviour between male and female *Drosophila*. In addition, the effect of mutations in the regulated genes could be tested in other behavioural paradigms.

Finally, further study of the roles of *hiw* and *hsp26* in neuronal adaptation to ethanol together with elucidation of the mechanisms underlying their transcriptional regulation in response to acute or chronic doses of ethanol in different brain regions could provide a molecular framework for understanding the development of tolerance and dependence in alcoholics.

This thesis has succeeded in showing that the use of microarray analysis coupled with behavioural genetics methodologies constitute an efficient approach in defining answers to the genetic and biological bases of the phenomena of alcohol sensitivity and the development of tolerance to the effects of alcohol that can lead to the complex problems of alcohol dependence and addiction.

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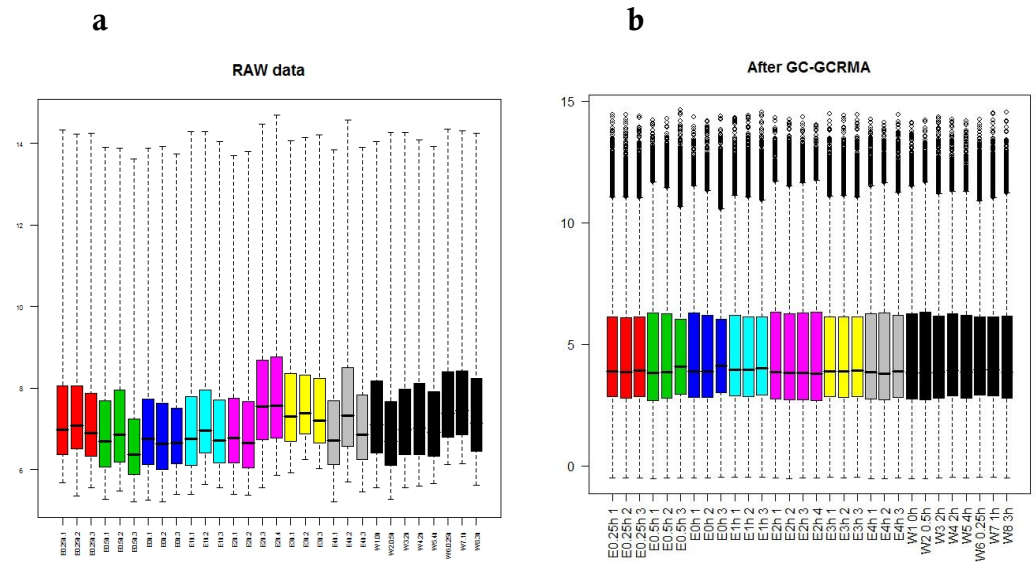
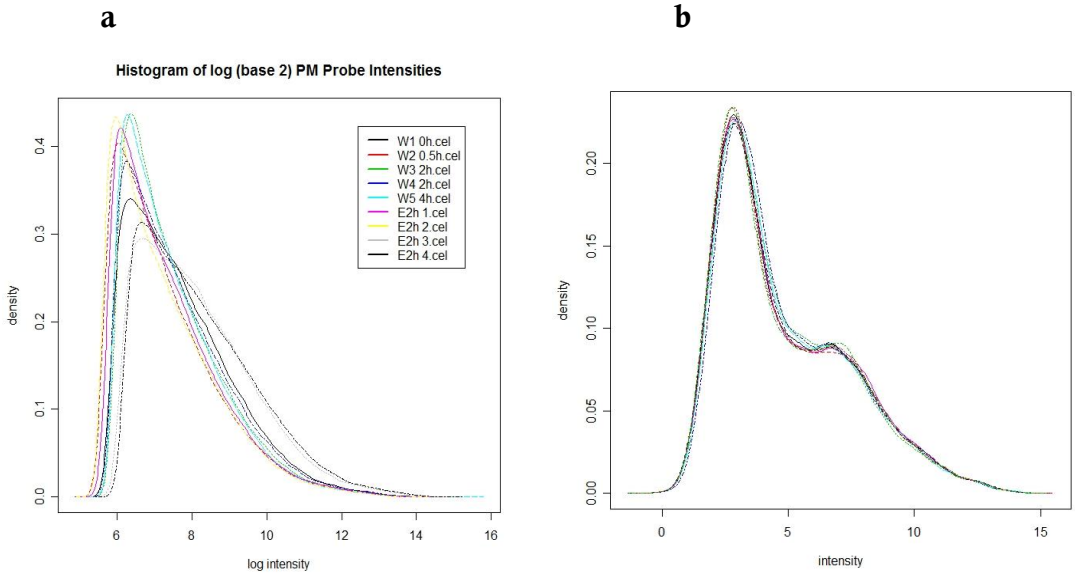
Appendix A.

Supplementary data in Figures.



A.1. Boxplots showing GCRMA normalised pooled time course array data.

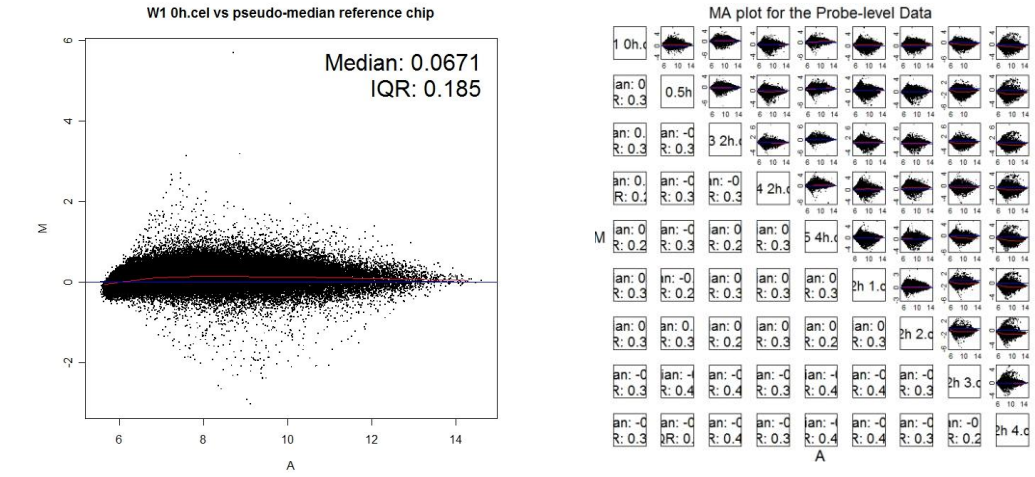
Shown are the pooled time course data before (a) and after (b) normalisation with GCRMA algorithm. One can see that the GCRMA normalised raw data shown in (b) display more variability when compared with the RMA normalisation for the same data shown in Figure 3.13 b of the Gene Expression Analysis in Chapter 3. The GCRMA normalisation was therefore not used on the pooled time course data.



A.2. Histograms of the 2 h microarray data.

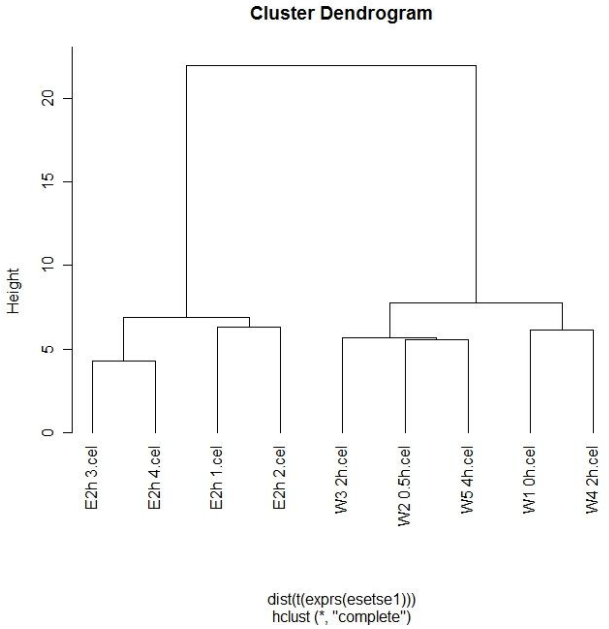
Shown are the data before (a) and after (b) normalisation with GCRMA algorithm. One can see that these arrays are aligned together and equally distributed in (b).

a **b**

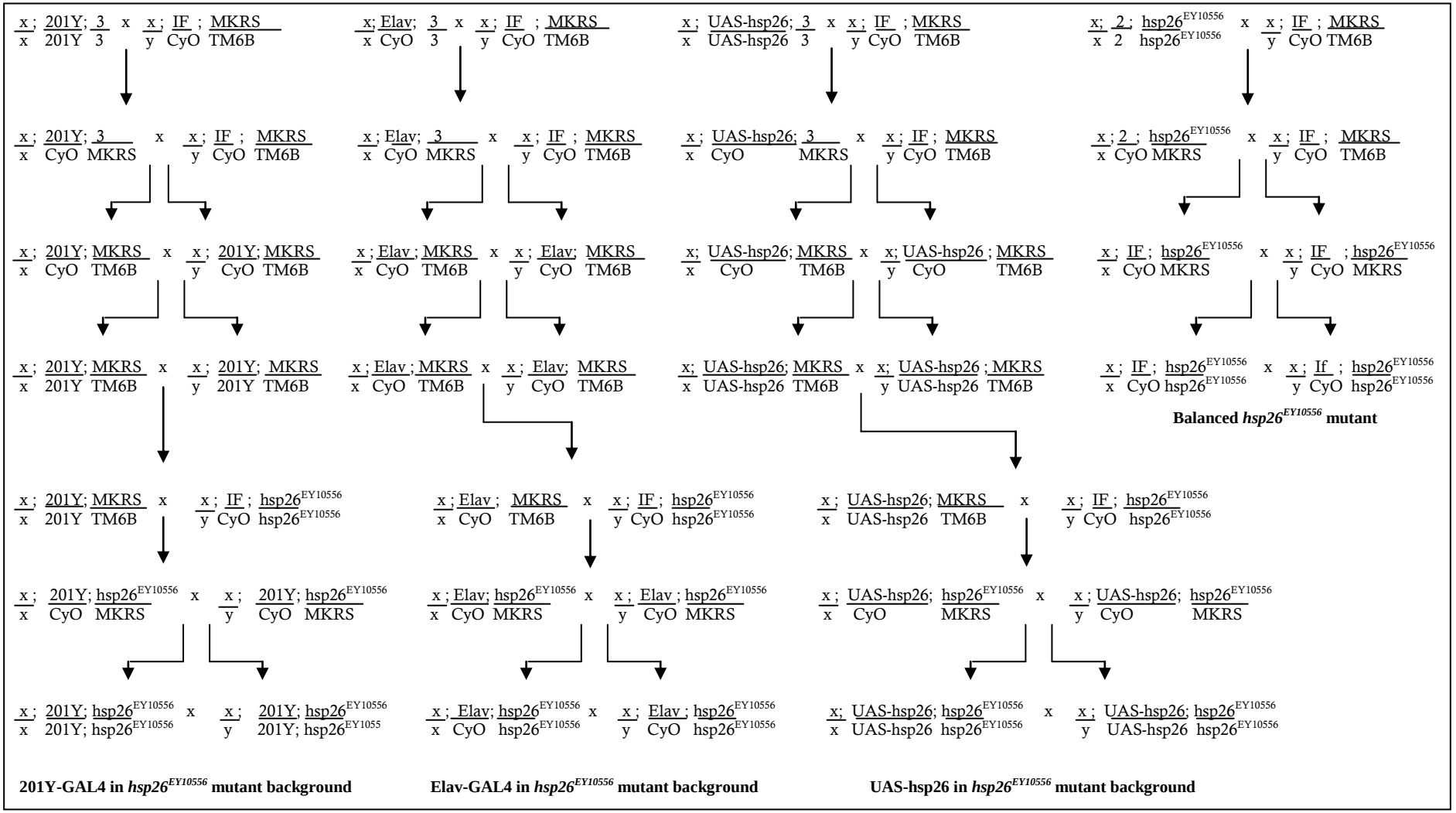


A.3. MA plots of the 2 h microarray data.

(a) MA plot of an array (W1 0h.cel) against the synthetic median array and **(b)** Pair wise MA comparison of all the nine arrays. The MvA points in both centred around zero. A loess regression line is added to the plots. Quality problems are most apparent from an MA-plot in a situation where the loess smoother oscillates wildly or if the variability of the M values appears to be greater in one or more of these nine arrays relative to the others (Bolstad et al., 2005). These anomalies did not occur in these plots.



A.4. Hierarchical cluster of the 2 h microarray data. A complete linkage cluster dendogram reveal a completely different pattern of expression between the ethanol treated (**E** cels) and the control (**W** cels) samples for the 155 gene set obtained using the stringent analysis (i.e. P-0.05 list of genes).



A.5. Genetic crosses used to create P[GAL4] lines 201Y and Elav and the P[UAS-hsp26] in *hsp26*^{EY10556} mutant background.

Appendix B.

Supplementary data in Tables.

B.1. Functional clusters of ethanol sensitive genes for upregulated genes

Category	Functional theme	GO annotation term	N	n	*P*
GOTERM_BP_ALL		protein folding		23	5.09E-16
GOTERM_BP_ALL		response to biotic stimulus		31	7.77E-11
GOTERM_BP_ALL		defense response		30	2.20E-10
GOTERM_BP_ALL	Response to stimulus	response to heat	51	10	1.45E-08
GOTERM_BP_ALL		response to temperature stimulus		10	6.36E-08
GOTERM_BP_ALL		response to abiotic stimulus		23	7.74E-07
GOTERM_BP_ALL		response to stress		20	1.94E-06
GOTERM_BP_ALL		response to stimulus		41	4.22E-06
GOTERM_BP_ALL		protein folding		23	5.09E-16
GOTERM_MF_ALL		unfolded protein binding		17	2.07E-14
GOTERM_CC_ALL		chaperonin-containing T-complex		7	1.26E-10
GOTERM_MF_ALL		adenyl nucleotide binding		33	6.31E-08
GOTERM_MF_ALL		purine nucleotide binding		36	2.75E-07
GOTERM_MF_ALL		ATP binding		31	3.01E-07
GOTERM_MF_ALL	Protein folding	nucleotide binding	65	39	3.74E-07
GOTERM_MF_ALL		ATPase activity		17	1.23E-04
GOTERM_MF_ALL		pyrophosphatase activity		20	2.55E-04
GOTERM_MF_ALL		ATPase activity, coupled		15	3.13E-04
GOTERM_MF_ALL		hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides		20	3.58E-04
GOTERM_MF_ALL		hydrolase activity, acting on acid anhydrides		20	3.65E-04
GOTERM_CC_ALL		cytosol		15	3.95E-04
GOTERM_MF_ALL		nucleoside-triphosphatase activity		19	5.78E-04
GOTERM_MF_ALL		glutathione transferase activity		9	3.30E-08
GOTERM_MF_ALL		transferase activity, transferring alkyl or aryl (other than methyl) groups		9	1.59E-06
GOTERM_BP_ALL	Defense response	response to toxin	32	11	1.60E-05
GOTERM_BP_ALL		response to chemical stimulus		15	3.61E-04
GOTERM_BP_ALL		oxygen and reactive oxygen species metabolism		7	9.39E-04
GOTERM_BP_ALL		protein metabolism		63	1.13E-04
GOTERM_BP_ALL		cellular protein metabolism		60	1.81E-04
GOTERM_BP_ALL		cellular macromolecule metabolism		61	3.47E-04
GOTERM_BP_ALL		metabolism		114	5.06E-04
GOTERM_BP_ALL	Protein metabolism	cellular metabolism	142	105	1.28E-03
GOTERM_BP_ALL		macromolecule metabolism		74	1.01E-02
GOTERM_BP_ALL		primary metabolism		99	1.05E-02
GOTERM_BP_ALL		cellular physiological process		129	1.05E-02
GOTERM_BP_ALL		cellular process		137	1.34E-02
GOTERM_BP_ALL		physiological process		135	5.92E-02

Category	Functional theme	GO annotation term	N	n	*P*
GOTERM_BP_ALL		actin filament organization		7	3.58E-04
GOTERM_BP_ALL		actin cytoskeleton organization and biogenesis		8	1.54E-03
GOTERM_BP_ALL		actin filament-based process		8	1.62E-03
GOTERM_BP_ALL	Cytoskeleton organisation	regulation of cell shape	25	7	2.57E-03
GOTERM_BP_ALL		actin polymerization and/or depolymerization		3	4.27E-02
GOTERM_BP_ALL		cytoskeleton organization and biogenesis		13	5.05E-02
GOTERM_BP_ALL		cellular morphogenesis		11	8.60E-02
GOTERM_BP_ALL		organelle organization and biogenesis		19	9.59E-02
GOTERM_BP_ALL		enzyme linked receptor protein signaling pathway		11	5.03E-04
GOTERM_BP_ALL		transmembrane receptor protein tyrosine kinase signaling pathway		9	1.10E-03
GOTERM_BP_ALL		torso signaling pathway		5	1.88E-03
GOTERM_BP_ALL		epidermal growth factor receptor signaling pathway		4	2.22E-02
GOTERM_BP_ALL		regulation of signal transduction		6	2.50E-02
GOTERM_BP_ALL		regulation of epidermal growth factor receptor signaling pathway		3	2.70E-02
GOTERM_BP_ALL		axis specification		7	4.86E-02
GOTERM_BP_ALL		protein kinase cascade		6	4.88E-02
GOTERM_BP_ALL	Signaling	regional subdivision	31	4	5.07E-02
GOTERM_BP_ALL		determination of anterior/posterior axis, embryo		4	5.07E-02
GOTERM_BP_ALL		photoreceptor cell differentiation (sensu Endopterygota)		5	5.93E-02
GOTERM_BP_ALL		Ras protein signal transduction		3	6.09E-02
GOTERM_BP_ALL		segmentation		6	6.86E-02
GOTERM_BP_ALL		pattern specification		9	7.20E-02
GOTERM_BP_ALL		embryonic pattern specification		6	7.44E-02
GOTERM_BP_ALL		embryonic axis specification		4	7.96E-02
GOTERM_BP_ALL		cell surface receptor linked signal transduction		17	8.84E-02
GOTERM_BP_ALL		photoreceptor cell differentiation		5	9.02E-02
GOTERM_MF_ALL		ligase activity, forming carbon-oxygen bonds		5	9.42E-03
GOTERM_MF_ALL		aminoacyl-tRNA ligase activity		5	9.42E-03
GOTERM_BP_ALL		translation		9	9.49E-03
GOTERM_BP_ALL		tRNA aminoacylation		5	1.34E-02
GOTERM_BP_ALL	RNA processing	tRNA aminoacylation for protein translation	26	5	1.34E-02
GOTERM_BP_ALL		amino acid activation		5	1.41E-02
GOTERM_MF_ALL		ligase activity		11	3.92E-02
GOTERM_BP_ALL		carboxylic acid metabolism		12	4.97E-02
GOTERM_BP_ALL		organic acid metabolism		12	4.97E-02
GOTERM_BP_ALL		tRNA metabolism		5	5.59E-02

Category	Functional theme	GO annotation term	N	n	*P*
GOTERM_BP_ALL		embryonic development		18	3.00E-04
GOTERM_BP_ALL		eye-antennal disc morphogenesis		9	6.70E-03
GOTERM_BP_ALL		eye-antennal disc development		9	8.72E-03
GOTERM_BP_ALL		nervous system development		17	1.44E-02
GOTERM_BP_ALL		eye development (sensu Endopterygota)		9	1.44E-02
GOTERM_BP_ALL		eye development		9	1.84E-02
GOTERM_BP_ALL		larval or pupal development (sensu Insecta)		13	3.15E-02
GOTERM_BP_ALL		compound eye development (sensu Endopterygota)		7	3.18E-02
GOTERM_BP_ALL		compound eye morphogenesis (sensu Endopterygota)		7	3.18E-02
GOTERM_BP_ALL		female gamete generation		13	3.24E-02
GOTERM_BP_ALL		post-embryonic development		13	3.73E-02
GOTERM_BP_ALL		oogenesis (sensu Insecta)		12	4.21E-02
GOTERM_BP_ALL	Reproduction	system development	40	18	4.24E-02
GOTERM_BP_ALL		eye morphogenesis (sensu Endopterygota)		7	4.44E-02
GOTERM_BP_ALL		gametogenesis		16	4.96E-02
GOTERM_BP_ALL		oogenesis		12	5.11E-02
GOTERM_BP_ALL		imaginal disc morphogenesis		10	5.18E-02
GOTERM_BP_ALL		eye morphogenesis		7	5.29E-02
GOTERM_BP_ALL		photoreceptor cell differentiation (sensu Endopterygota)		5	5.93E-02
GOTERM_BP_ALL		sexual reproduction		16	5.94E-02
GOTERM_BP_ALL		metamorphosis (sensu Insecta)		10	6.46E-02
GOTERM_BP_ALL		metamorphosis		10	6.65E-02
GOTERM_BP_ALL		segmentation		6	6.86E-02
GOTERM_BP_ALL		embryonic pattern specification		6	7.44E-02
GOTERM_BP_ALL		cellular morphogenesis		11	8.60E-02
GOTERM_BP_ALL		organ development		18	8.61E-02
GOTERM_BP_ALL		photoreceptor cell differentiation		5	9.02E-02

Microarray gene expression screening of alcohol and control cases using multiple testing correction of Benjamini and Hochberg's false discovery rate method (fdr $\leq$ 0.1) and overlaps of differentially expressed genes across two alternative algorithms of RMA and GCRMA identified genes sensitive to acute ethanol exposure in *Drosophila*. Upregulated genes were clustered into functional themes by the Database for Annotation, Visualisation and Integrated Discovery (DAVID) software (Dennis et al., 2003). Annotation terms reaching significant enrichment scores (EASE score $\leq$ 0.1) are reported. Clusters containing less than 3 genes were removed to avoid overestimation of the true size of the functional theme. N: number of genes in functional theme, n: number of genes with enriched annotation term for all differentially expressed genes clustered by DAVID.

B.2. Functional clusters of ethanol sensitive genes for downregulated genes

Category	Functional theme	GO annotation term	N	n	*P*
GOTERM_MF_ALL	Oxidoreductase activity	oxidoreductase activity	36	17	3.28E-05
GOTERM_BP_ALL		electron transport		12	1.92E-04
GOTERM_BP_ALL		generation of precursor metabolites and energy		13	1.26E-03
GOTERM_MF_ALL		transporter activity		18	8.05E-03
GOTERM_BP_ALL		transport		25	1.26E-02
GOTERM_BP_ALL		localization		27	2.25E-02
GOTERM_BP_ALL		establishment of localization		26	2.91E-02
GOTERM_BP_ALL	Lipid metabolism	lipid metabolism	12	12	1.52E-03
GOTERM_BP_ALL		cellular lipid metabolism		9	4.37E-03
GOTERM_BP_ALL		fatty acid metabolism		5	6.70E-03
GOTERM_MF_ALL	Catalytic activity	acyl-CoA dehydrogenase activity	8	3	4.05E-03
GOTERM_BP_ALL		fatty acid metabolism		5	6.70E-03
GOTERM_BP_ALL		acyl-CoA metabolism		3	7.56E-03
GOTERM_MF_ALL		oxidoreductase activity, acting on the CH-CH group of donors		3	1.78E-02
GOTERM_BP_ALL	Pigmentation	pigmentation	8	5	5.24E-04
GOTERM_BP_ALL		pigmentation during development		5	5.24E-04
GOTERM_BP_ALL		eye pigmentation		4	1.52E-03
GOTERM_BP_ALL		aromatic compound metabolism		6	7.00E-03
GOTERM_BP_ALL		secondary metabolism		4	1.99E-02
GOTERM_BP_ALL		pigment biosynthesis		3	5.24E-02
GOTERM_BP_ALL		pigment metabolism		3	6.52E-02
GOTERM_BP_ALL		heterocycle metabolism		4	9.60E-02
GOTERM_MF_ALL	Metabolism	catalytic activity	76	51	1.22E-05
GOTERM_BP_ALL		metabolism		58	2.50E-03
GOTERM_BP_ALL		primary metabolism		53	4.09E-03
GOTERM_BP_ALL		physiological process		69	1.84E-02
GOTERM_BP_AL	Transport activity	generation of precursor metabolites and energy	23	13	1.26E-03
GOTERM_MF_ALL		monooxygenase activity		6	1.80E-03
GOTERM_MF_ALL		electron transporter activity		6	2.19E-02
GOTERM_CC_ALL		membrane fraction		4	2.47E-02
GOTERM_CC_ALL		cell fraction		4	2.67E-02
GOTERM_MF_ALL		iron ion binding		4	7.42E-02
GOTERM_BP_ALL	Fatty acid metabolism	fatty acid metabolism	9	5	6.70E-03
GOTERM_BP_ALL		fatty acid beta-oxidation		3	1.01E-02
GOTERM_BP_ALL		fatty acid oxidation		3	1.10E-02
GOTERM_BP_ALL	Organic acid metabolism	carboxylic acid metabolism	13	10	3.12E-03
GOTERM_BP_ALL		organic acid metabolism		10	3.12E-03
GOTERM_BP_ALL		aromatic compound metabolism		6	7.00E-03
GOTERM_BP_ALL		aromatic amino acid family metabolism		3	1.10E-02

Category	Functional theme	GO annotation term	N	n	*P*
GOTERM_MF_ALL		porter activity		7	4.88E-03
GOTERM_MF_ALL		electrochemical potential-driven transporter activity		7	4.88E-03
GOTERM_MF_ALL	Porter activity	transporter activity	22	18	8.05E-03
GOTERM_MF_ALL		solute:sodium symporter activity		3	8.37E-02
GOTERM_MF_ALL		solute:cation symporter activity		3	8.56E-02
GOTERM_BP_ALL		imaginal disc morphogenesis		6	8.26E-02
GOTERM_BP_ALL	Morphogenesis	metamorphosis (sensu Insecta)	28	6	9.52E-02
GOTERM_BP_ALL		larval or pupal development (sensu Insecta)		7	9.69E-02
GOTERM_BP_ALL		metamorphosis		6	9.70E-02

Microarray gene expression screening of ethanol and control cases using multiple testing correction of Benjamini and Hochberg's false discovery rate method (fdr $\leq$ 0.1) and overlaps of differentially expressed genes across two alternative algorithms of RMA and GCRMA identified genes sensitive to acute ethanol exposure in *Drosophila*. Downregulated genes were clustered into functional themes by the Database for Annotation, Visualisation and Integrated Discovery (DAVID) software (Dennis et al., 2003). Annotation terms reaching significant enrichment scores (EASE score $\leq$ 0.1) are reported. Clusters containing less than 3 genes were removed to avoid overestimation of the true size of the functional theme. N: number of genes in functional theme, n: number of genes with enriched annotation term for all differentially expressed genes clustered by DAVID.

B.3. Genes common in three microarray studies of EtOH regulation in *Drosophila*.

Flybase ID	Gene symbol	Name	Function
FBgn0034628	*Acox57D-p*	*acyl-Coenzyme A oxidase at 57D proximal*	acyl-CoA oxidase activity
FBgn0011746	*ana*	*anachronism*	protein binding
FBgn0034494	*CG10444*	*CG10444*	sodium-dependent multivitamin transmembrane transporter activity
FBgn0036334	*CG11267*	*CG11267*	unfolded protein binding
FBgn0039330	*CG11909*	*CG11909*	alpha-glucosidase activity
FBgn0031069	*CG12703*	*CG12703*	transporter activity
FBgn0032961	*CG1416*	*CG1416*	ATPase activator activity
FBgn0031037	*CG14207*	*CG14207*	unknown
FBgn0039620	*CG1443*	*CG1443*	binding
FBgn0035734	*CG14823*	*CG14823*	lysozyme activity
FBgn0034394	*CG15096*	*CG15096*	sodium symporter activity
FBgn0086691	*CG15261*	*UK114*	unknown
FBgn0029766	*CG15784*	*CG15784*	unknown
FBgn0033188	*CG1600*	*CG1600*	oxidoreductase activity; zinc ion binding
FBgn0040972	*CG16978*	*CG16978*	unknown
FBgn0032029	*CG17292*	*CG17292*	triacylglycerol lipase activity
FBgn0038347	*CG18522*	*CG18522*	oxidoreductase activity; iron ion binding
FBgn0033204	*CG2065*	*CG2065*	binding; oxidoreductase activity
FBgn0031668	*CG31917*	*CG31917*	unknown
FBgn0023507	*CG3835*	*CG3835*	oxidoreductase activity; FAD binding
FBgn0023507	*CG4199*	*CG4199*	oxidoreductase activity; FAD binding
FBgn0039073	*CG4408*	*CG4408*	metallocarboxypeptidase activity
FBgn0032160	*CG4598*	*CG4598*	dodecenoyl-CoA delta-isomerase activity
FBgn0034909	*CG4797*	*CG4797*	glucose transmembrane transporter activity
FBgn0030817	*CG4991*	*CG4991*	amino acid transmembrane transporter activity
FBgn0035950	*CG5288*	*CG5288*	galactokinase activity
FBgn0038516	*CG5840*	*CG5840*	pyrroline-5-carboxylate reductase activity
FBgn0038730	*CG6300*	*CG6300*	long-chain fatty acid transporter activity
FBgn0036262	*CG6910*	*CG6910*	inositol oxygenase activity; iron ion binding
FBgn0038631	*CG7695*	*CG7695*	unknown
FBgn0030809	*CG9086*	*CG9086*	ubiquitin-protein ligase activity
FBgn0030332	*CG9360*	*CG9360*	oxidoreductase activity
FBgn0032076	*CG9510*	*CG9510*	argininosuccinate lyase activity

Flybase ID	Gene symbol	Name	Function
FBgn0039759	*CG9733*	*CG9733*	serine-type endopeptidase activity
FBgn0027842	*CPTI*	*mitochondrial carnitine palmitoyltransferase I*	carnitine O-palmitoyltransferase activity
FBgn0038681	*Cyp12a4*	*Cyp12a4*	electron carrier activity
FBgn0037817	*Cyp12e1*	*Cyp12e1*	electron carrier activity
FBgn0033304	*Cyp6a13*	*Cyp6a13*	electron carrier activity
FBgn0004629	*Cys*	*Cystatin-like*	cysteine-type endopeptidase inhibitor activity
FBgn0026718	*fu12*	*fu12*	1-acylglycerol-3-phosphate O-acyltransferase activity
FBgn0001149	*GstD1*	*Glutathione S transferase D1*	glutathione transferase activity
FBgn0010038	*GstD2*	*Glutathione S transferase D2*	glutathione transferase activity
FBgn0034335	*GstE1*	*Glutathione S transferase E1*	glutathione transferase activity
FBgn0063495	*GstE5*	*Glutathione S transferase E5*	glutathione transferase activity
FBgn0063494	*GstE6*	*Glutathione S transferase E6*	glutathione transferase activity
FBgn0063493	*GstE7*	*Glutathione S transferase E7*	glutathione transferase activity
FBgn0010228	*HmgZ*	*HMG protein Z*	DNA binding
FBgn0001224	*Hsp23*	*Heat shock protein 23*	actin binding
FBgn0015245	*Hsp60*	*Heat shock protein 60*	unfolded protein binding
FBgn0010053	*Jheh1*	*Juvenile hormone epoxide hydrolase 1*	juvenile hormone epoxide hydrolase activity
FBgn0011296	*l(2)efl*	*lethal (2) essential for life*	unknown
FBgn0034389	*Mctp*	*Multiple C2 domain and transmembrane region protein*	unknown
FBgn0002719	*Men*	*Malic enzyme*	malate dehydrogenase activity
FBgn0002789	*Mp20*	*Muscle protein 20*	calcium ion binding; actin binding
FBgn0039684	*Obp99d*	*Odorant-binding protein 99d*	odorant binding
FBgn0004654	*Pgd*	*Phosphogluconate dehydrogenase*	phosphogluconate dehydrogenase activity
FBgn0003231	*ref(2)P*	*refractory to sigma P*	zinc ion binding
FBgn0030318	*rho-4*	*rhomboid-4*	serine-type peptidase activity
FBgn0038257	*smp-30*	*Senescence marker protein-30*	unknown
FBgn0028990	*Spn27A*	*Serpin-27A*	serine-type endopeptidase inhibitor activity

Flybase ID	Gene symbol	Name	Function
FBgn0041182	*TepII*	*Thiolester containing protein II*	peptidase inhibitor activity
FBgn0004924	*Top1*	*Topoisomerase 1*	DNA topoisomerase activity
FBgn0021872	*Xbp1*	*X box binding protein-1*	transcription factor activity
FBgn0040064	*yip2*	*yippee interacting protein 2*	acetyl-CoA C-acyltransferase activity

Comparison of genes obtained from Morozova et al., 2006 and 2007 with the 155 genes obtained from the stringent analysis (i.e. P-0.05 list of genes) in this study produced 64 gene overlaps.