THE EFFECTS OF ALCOHOL ON G PROTEIN GENE EXPRESSION IN *DROSOPHILA MELANOGASTER*

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Abstract

Alcohol is one of the most widely used and socially acceptable drugs in the world. However, its chronic use can lead to serious problems including the development of tolerance. Acute and chronic use of ethanol leads to short-term and long-term changes in gene expression in the brain resulting in cellular and molecular adaptations that are associated with addictive behaviours. Our understanding of the mechanisms by which alcohol produces these changes in the brain is not fully understood. Ethanol affects the function of receptors including G protein-coupled receptors that activate heterotrimeric G proteins. The aim of this thesis is to understand whether ethanol can cause changes in G protein gene expression using *Drosophila melanogaster* as a model.

Drosophila is a genetically tractable organism suitable to investigate the neural substrates of neuroadaptive responses to ethanol. The response to ethanol and the onset of tolerance was measured in wild-type and mutant *Drosophila*. While tolerance was consistently observed in all fly populations, individual differences in sensitivity to alcohol were observed, which prompted the isolation of subpopulations of *Drosophila* with distinct ethanol characteristics. Relative mRNA expression in G protein subunits was measured using quantitative real-time polymerase chain reaction in different *Drosophila* strains (wild-type, subpopulations of early and late responders, G protein mutants and dopamine 1-like D2 receptor mutants) that have received zero, one, two or three ethanol exposures at 24 h intervals.

When measured in the wild-type strains, changes in G protein subunits expression were variable. However in a subpopulations of early responders that were selected for high ethanol sensitivity, a non-statistically significant decrease of two Gα-protein subunits: G_i and G_q were observed. When measured in two *Drosophila* mutant strains, flies with

either deletion of dopamine D2 receptor or a mutated G_i gene subunit, statistically significant changes were observed in G_i and G_q subunits. In a further study, a mutant expressing non-functional G_q, the G_i expression was not affected by the ethanol treatment suggesting a possible crosstalk between different signalling pathways. These results justify a more detailed investigation of changes in G protein subunits following acute and chronic exposure to ethanol in *Drosophila*, which will allow verifying the hypothesis that changes in gene expression of G proteins participate in addictive behaviours in *Drosophila*. These findings in *Drosophila*, which share genetic and functional characteristics with the mammalian nervous system, could translate into important advances in identifying targets for treatment for alcohol addiction in humans.

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Abbreviations

µg	Microgram
µl	Microlitre
M	Molar
AC	Adenylyl cyclase
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
bp	Base pairs
cAMP	Cyclic adenosine 3', 5' monophosphate
CREB	cAMP-responsive binding protein
CO_2	Carbon dioxide
CNS	Central nervous system
DNA	Deoxyribonucleic acid
Dop1R2	Dopamine 1-like D2 receptor
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
GABA	γ-aminobutyric acid
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
mRNA	Messenger RNA
miRNA	Micro RNA
NAc	Nucleus accumbens
NCBI	National centre for Biotechnology
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-$_D$-aspartate
NPY	Neuropeptide Y
OD	Optical density
PE	Protein extraction
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
SEM	Standard Error Mean
TBST	Tris buffered saline-tween

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Chapter 1: Literature Review

1.1. Alcohol

Alcohols are organic compounds containing a hydroxyl group attached to a carbon atom (Most *et al*., 2014). Ethanol (Figure 1.1), the psychoactive constituent of alcoholic beverages, has been consumed recreationally for tens of thousands of years (Hanson, 1995) and can produce both positive mood states and stress relieving properties (Spanagel, 2009). For the remainder of this thesis, the terms alcohol and ethanol will be used interchangeably. Ethanol is known to act directly on the central nervous system (CNS) to produce changes in mood and behaviour. However, the mechanisms by which ethanol exerts its CNS effects are yet to be fully elucidated. Ethanol can be easily distinguished from other psychoactive drugs by the fact that it is both a source of metabolic energy and a psychoactive molecule and by the fact that ethanol's actions in the brain are not initiated by binding to its own specific receptors (Tabakoff, 1996).

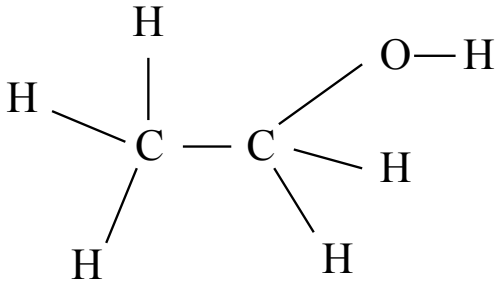

Figure 1.1. Ethanol structure

1.1.1. Alcohol absorption and metabolism

Alcohol, when consumed, is absorbed into the bloodstream through the stomach and intestines, and it readily crosses the blood-brain barrier (Most *et al*., 2014). Metabolism of alcohol requires a number of processes, one of which is known as oxidation. Over 90% of the absorbed alcohol is metabolized in the body through oxidative pathways and the majority of this metabolism takes place in the liver (Tabakoff, 1996). The remainder is eliminated in an unchanged form through non-oxidative pathways that occur mainly in extrahepatic tissues (Zakhari, 2013).

The first step of the oxidative pathway is the oxidation of alcohol to acetaldehyde, a highly reactive and toxic by-product that may contribute to tissue damage and possibly addictive processes. The reaction is catalysed by the enzyme alcohol dehydrogenase (ADH), a dimeric protein found predominately in the cytoplasm of liver cells (Zakhari, 2006; Zakhari, 2013) (Figure1.2). Different ADH types can be divided into five classes based on their subunit and isoenzyme (variants) composition (Agarwal, 2001). The oxidation of alcohol to acetaldehyde is regulated by an intermediate carrier of electrons, nicotinamide adenine dinucleotide (NAD^+) as a cofactor (coenzyme), which reduced by two electrons to form NADH (reduced form of NAD^+)(Zakhari, 2006).

The second pathway in alcohol metabolism comprises of the microsomal ethanol-oxidizing system (MEOS), which is located in the smooth endoplasmic reticulum and involves the enzyme cytochrome P450 (Manzo-Avalos and Saavedra-Molina, 2010). This system accounts for the major non-ADH ethanol metabolic pathway in the liver (Lieber, 2000). In humans, various cytochrome P450 isoenzymes, including CYP2E1, 1A2 and 3A4, have been reported (Agarwal, 2001) but have not been associated to susceptibility to alcoholism or alcohol-induced organ damage (Nagy, 2004). CYP2E1 is induced by chronic alcohol consumption and plays a significant role in the metabolism

of ethanol to acetaldehyde. In addition, CYP2E1-dependent ethanol oxidation may occur in other tissues such as the brain, where ADH activity is low. CYP2E1 also produces several reactive oxygen species (ROS), including hydroxyethyl, superoxide anion, and hydroxyl radicals, which increases the risk of tissue damage (Zakhari, 2006).

The third pathway involves the peroxisomal oxidation of ethanol and involves the enzyme, catalase. Catalase is capable of oxidizing ethanol *in vitro* in the presence of a hydrogen peroxide (H_2O_2) generating system, such as the enzyme complex nicotinamide adenine dinucleotide phosphate oxidase or the enzyme, xanthine oxidase, into acetaldehyde and water (Cederbaum, 2012; Zakhari, 2006). This however, plays an insignificant role in alcohol oxidation (Cederbaum, 2012). Acetaldehyde is a highly toxic and reactive by-product to the hepatocytes that may contribute to tissue damage because it forms adducts to protein and DNA that aid lipid peroxidation, glutathione depletion, and mitochondrial damage (Nassir and Ibdah, 2014). It also contributes to the changes in the redox state of the cell and the formation of reactive oxygen species (ROS) (Zakhari, 2013). The acetaldehyde produced from ADH and the peroxisomal pathway, is rapidly metabolized to acetate by the enzyme aldehyde dehydrogenase (ALDH) in humans, utilizing NAD^+ as cofactor to form acetate and NADH. Acetate is then metabolized to acetyl coenzyme A in the tricarboxylic cycle (Figure 1.2.) (Zakhari, 2006).

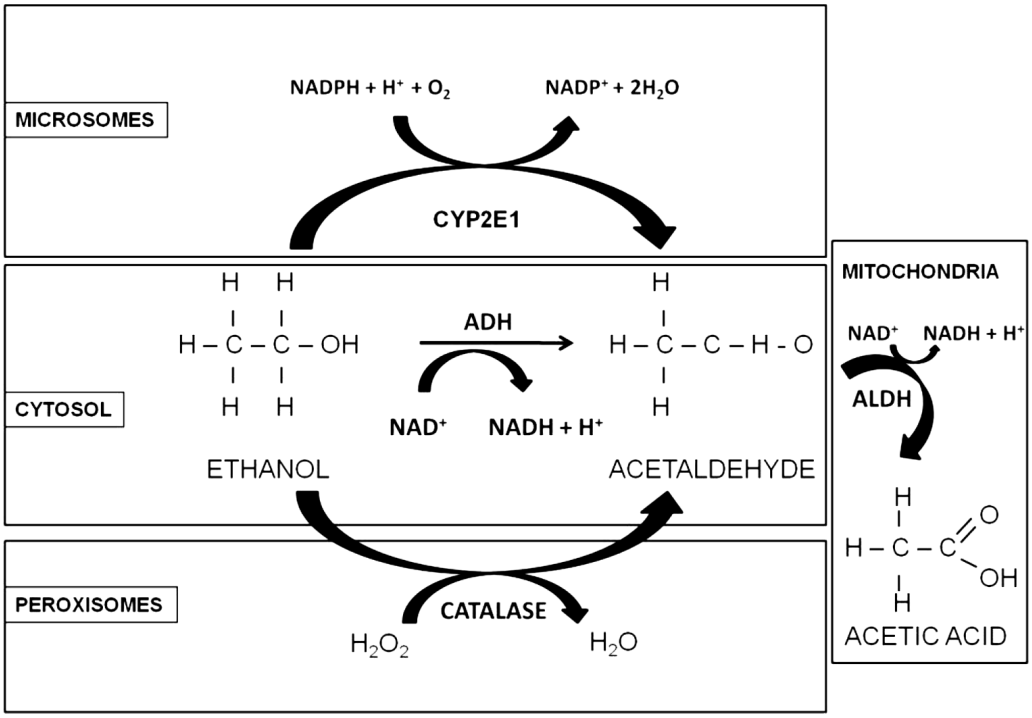

Figure 1.2. Alcohol metabolism

The oxidative pathways involved in the metabolism of alcohol. The enzymes, alcohol dehydrogenase (ADH), cytochrome P450 (CYP2E1) and catalase, all contribute to the oxidative metabolism of alcohol. ADH converts ethanol to acetaldehyde in the cytosol of the cell. This reaction involves nicotinamide adenine dinucleotide (NAD^+), which is reduced by two electrons to form NADH. In the microsomes, CYP2E1 metabolizes alcohol to acetaldehyde at chronic ethanol concentrations. Catalase, located in the peroxisomes, requires hydrogen peroxide to oxidize alcohol. Acetaldehyde is metabolized mainly by aldehyde dehydrogenase (ALDH2) in the mitochondria to form acetate and NADH. (With permission from Manzo-Avalos and Saavedra-Molina, 2010).

1.1.2. Alcohol abuse and addiction

Many consumers lose control over their intake of alcohol, with enormous health and socioeconomic impacts on the world population. Excessive alcohol intake can produce a multiplicity of medical complications to the liver, cardiovascular system, gonads and the brain (Spanagel *et al*., 1996). Alcohol-induced brain damage is a particular problem in pregnancy, resulting in fetal alcohol syndrome, which represents the most common form of acquired mental disability, affecting 7/1,000 infants (Niccols, 2007). During adolescence, the consequences of alcohol drinking, especially binge drinking, on organ

dysfunction and damage are largely unknown. The adolescent brain displays higher sensitivity to alcohol-induced brain damage and cognitive impairment than the adult brain, in humans as well as in rodents (Crews *et al*., 2000). Furthermore, the onset of alcohol use during adolescence leads to a higher susceptibility to stress-induced alcohol consumption (Siegmund *et al*., 2005) and a greater risk of developing alcohol addiction in adulthood (Grant and Dawson, 1997).

Alcohol use and abuse entails serious societal and economic fallout in the form of criminality, decreased productivity and increased health costs (Spanagel, 2009). Alcohol abuse has a high comorbidity with other psychiatric disorders (Kessler *et al*., 1994). Due to the anxiolytic effects of alcohol, people who suffer from anxiety disorders and depression often use alcohol as a kind of self-medication but in most cases, the driving force of alcohol abuse is the development of an addictive behaviour (Spanagel, 2009). Alcohol addiction, also known as alcohol related disorder (*Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-V) in 2013) is characterized by repetitive alcohol drinking which leads to a loss of control over the consumption of alcohol (Moonat *et al*., 2010).

Some aspects of alcohol addiction can occur relatively swiftly in response to acute administration of alcohol, however, most changes in brain function associated with addiction occur gradually over time in response to prolonged alcohol exposure (Nestler *et al*., 1993; Most *et al*., 2014). These gradual developing changes can persist for a long time after cessation of chronic ethanol administration and are described as tolerance, sensitization, dependence and withdrawal (Nestler *et al*., 1993). Tolerance refers to ethanol-induced adaptations that lead to diminishing response to a constant ethanol dose (Chao and Nestler, 2004; Pietrzykowski and Treistman, 2008). Sensitization or reverse tolerance refers to ethanol-induced adaptations that increase ethanol responsiveness with repeated drug exposure (Chao and Nestler, 2004; Nestler *et al*., 1993). Dependence

describes the need for continued drug exposure to avoid withdrawal symptoms, which causes a significant negative affective state and some cases profound somatic abnormalities (Chao and Nestler, 2004).

The prominence of delayed, progressively developing and persistent adaptations in brain function during alcohol addiction suggests that long term changes in the brain are important in mediating addictive phenomena (Kaewsuk *et al*., 2001). Addiction processes can be thus viewed as a drug-induced neural plasticity and as such can serve as a model system to investigate the types of neurobiological mechanisms involved in plasticity (Nestler *et al*., 1993).

There are two major brain regions involved in drug addiction behaviours, the mesocorticolimbic dopaminergic system and the locus coreuleus (Nestler *et al*., 1994). The mesocorticolimbic dopaminergic system has been reported to be the key mediator in the rewarding effects of alcohol and it comprises of the ventral tegmental area and its targets, the nucleus accumbens and the amygdala (Flatscher-Bader *et al*., 2006). Molecular and cellular changes in the nucleus accumbens with acute and repeated alcohol exposure may underline certain aspects in the development of alcohol addiction (Moonat *et al*., 2010).

Due to the complexity of alcohol addictive processes, it cannot be modelled in animals as a whole (Vengeliene *et al*., 2008). However, the initiation and maintenance of alcohol consumption can be successfully mimicked or modelled in animal models (Segura and Spanagel, 2006) and the results obtained from these models provides an empirical framework for understanding the molecular basis of addiction (Koob *et al*., 1998). The underlying molecular and cellular changes that occur with the transition from occasional alcohol use to pathological abuse and addiction are only partially understood.

1.1.3. Genetics of alcohol addiction

Alcohol addiction is influenced by both genetic and environmental factors. Alcohol addiction tends to run in families and studies of twins, family, adoption, linkage and association suggest a genetically transmitted susceptibility for alcoholism (Diamond and Gordon, 1997). Twins studies demonstrated that the amount of alcohol one consumes has a genetic influence (Kendler *et al*., 2011). Identical twins have a significantly higher concordance of alcoholism than fraternal twins, even when environmental factors, such as the greater frequency of social contact between identical twins, are taken into account (Heath *et al*., 1989). The twins studies provide information on the genetic impact on addiction, which aspects of addiction are most heritable, whether the same genes are influencing disease in both genders, and whether multiple disease share any common genetic influences (Anderson *et al*., 2005). Schuckit (1994) reported young men with a positive family history for alcoholism have a diminished ataxia response after drinking a test dose of alcohol (Schuckit, 1994).

Adoption studies have provided evidence that alcoholism in the biological father predicts alcoholism in the children even when the child is raised by unrelated adoptive parents (Diamond and Messing, 1994; Sher, 1997). These types of studies provide some of the strongest evidence for heritability of alcoholism (Diamond and Gordon, 1997), and studies have reported reductions in alcoholism occurrence in consequent generation after removal from home with alcoholic fathers (Cloninger *et al*., 1981).

Linkage studies have also been employed to identifying candidate chromosomal regions susceptible to alcoholism. Studies have shown alcoholism is linked with the chromosome 4q blood group marker MNS and the esterase D marker on chromosome 13q (Hill *et al*., 1988; Tanna *et al*., 1988). Other studies implicated chromosomes 1, 2, 3, 7 and 8 in relation to alcoholism (Edenberg, 2002; Foroud *et al*., 2000).

Unlike linkage studies, association studies are able to accurately associate gene or genes contributing to a disorder or phenotype of interest (Dick and Foroud, 2002). This usually involves the analysis of candidate genes to test the association between a particular allele of a candidate gene and a specific behaviour. The candidate gene is selected based on its supposed role in the behaviour or other known information relating to the behavioural outcome (Dick and Foroud, 2002).

Recently, genome-wide association studies have been used to identify genes across chromosomes and pathways important in alcohol addiction (Edenberg and Foroud, 2014; Schuckit, 2014). In these studies, the association of a phenotype with hundreds of thousands of single nucleotide polymorphisms (SNPs) distributed throughout the genome is evaluated (Manolio, 2010). Previous study has reported an association of alcohol intake levels with a variation in the *autism susceptibility candidate 2 (AUTS2)* gene (Schumann *et al*., 2011). Other GWAS have highlighted associations to alcohol dependence, including NMDA-dependent AMPA receptor (Karpyak *et al*., 2012), alcohol-metabolizing genes (Edenberg, 2012; Jairam and Edenberg, 2014), *GABRA 2* gene (Bierut *et al*., 2010). There are no reports at the moment on the identification of G proteins by GWAS. In summary, GWAS approaches have highlighted some potentially promising genes that might contribute to alcohol drug-related problems.

1.1.4. Targets of alcohol

Ethanol is thought to exert its effects on cells by altering the physical properties of lipid bilayer membranes of the cells, however, a wide array of studies have reported that ethanol interacts with and modifies the functions of receptor proteins of the nervous system including ion channels and second messenger proteins (Harris, 1999; Peoples *et al*., 1996).

Due to the unspecific pharmacological nature of alcohol, ethanol is known to modulate the functions of several neurotransmitter receptors including N-methyl-D-aspartate (NMDA), γ-aminobutyric acid A ($GABA_A$), 5-hydroxytryptamine-3 ($5HT_3$), nicotinic acetylcholine (nAChR) receptors and voltage-dependent channels (Figure 1.3). Interaction of alcohol with these target proteins leads to changes in the activity of many enzymes and regulators of gene expression (Diamond and Gordon, 1997; Erdozain and Callado, 2014).

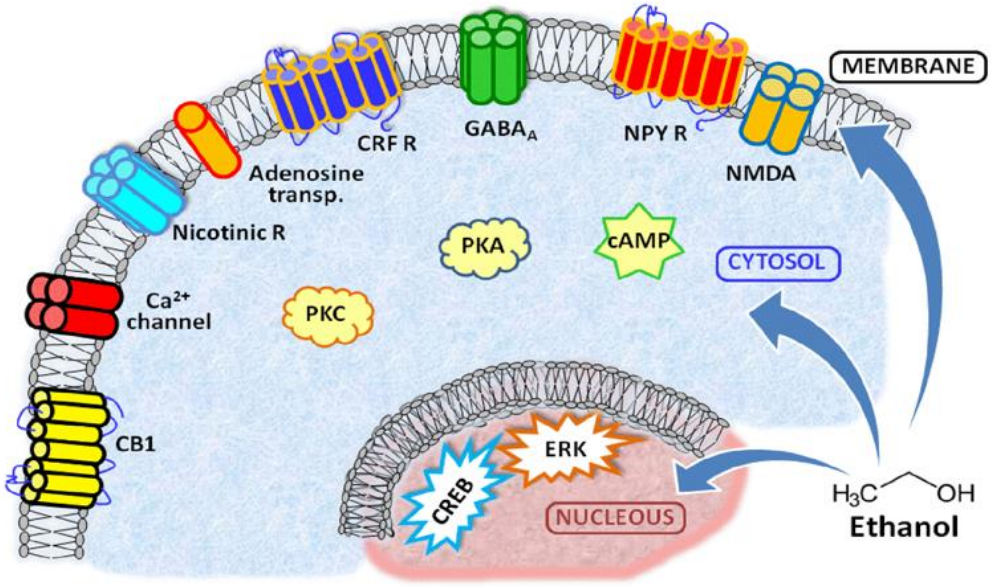

Figure 1.3. Representation of the number of targets that are modulated by ethanol directly or indirectly

Membrane receptors, signalling elements and transcription factors in the nucleus. R: receptor, NMDA: N-methyl-D-aspartate, NPY: neuropeptide Y, $GABA_A$: γ-aminobutyric acid A, CRF: corticotrophin-releasing factor, CB1: cannabinoid receptor 1, PKC: protein kinase C, PKA: protein kinase A, cAMP: cyclic adenosine monophosphate, CREB: cAMP-responsive binding protein, ERK: extracellular-signal-regulated kinase (With permission from Erdozain and Callado, 2014).

1.1.4.1. N-methyl-D-aspartate (NMDA) receptor

The main excitatory neurotransmitter in the brain is glutamate and it plays a vital role in the pharmacological effects of ethanol (Erdozain and Callado, 2014). NMDA receptors

are one of the major targets of research as they are implicated in learning and memory, long-term potentiation (LTP) and long-term depression (LTD) in models of synaptic plasticity (Diamond and Gordon, 1997). The NMDA receptor consists of four subunits, which form a cation channel. Glutamate binding to the NMDA receptor leads to increased permeability of calcium (Traynelis *et al*., 2010). The increase in calcium, initiates synaptic signalling by activating protein kinases, proteases and phosphatases (Diamond and Gordon, 1997).

Ethanol's binding sites on the NMDA receptor have not been identified and there have been reports that suggest ethanol exerts its effect on the NMDA receptor through protein kinase C (Li *et al*., 2005). Ethanol has also been discovered to inhibit other ionotropic glutamate receptors, kainate and AMPA receptors (Most *et al*., 2014). Lovinger *et al*., (1989) demonstrated that ethanol inhibits NMDA function in a concentration-dependent manner over the range of 5-50mM, which produces intoxication. Acute exposure to ethanol antagonizes NMDA receptors, altering many cellular functions such as inhibition of calcium flux into cells, which in turn inhibits the excitatory effect of glutamate-activated NMDA receptor function (Erdozain and Callado, 2014; Wirkner *et al*., 1999).

On the other hand, chronic exposure to ethanol in rats and in cultured cells, causes an up-regulation of NMDA receptors (Hoffman and Tabakoff, 1994). Similarly, an increase of NMDA receptors has also been observed in the brain of human alcoholic's subsets (Michaelis *et al*., 1990). This increase in NMDA receptor function is probably a compensatory change induced by the inhibitory effect of acute alcohol (Fadda and Rossetti, 1998). NMDA receptor activation is also implicated in withdrawal syndrome, including delirium tremens and especially seizures (Hughes, 2009).

1.1.4.2. γ-Aminobutyric acid A ($GABA_A$) receptor

γ-Aminobutyric acid A (GABA) is the main inhibitory neurotransmitter in the mammalian brain and mediates its effects via $GABA_A$ and $GABA_B$ receptors (Stephenson, 1995). Previous evidence suggested that GABA is associated in many ethanol-induced behaviours such as motor incoordination, sedation, withdrawal and alcohol preference (Kumar *et al*., 2009). For example, $GABA_B$ receptor 1 has been involved in behaviour-impaired effects of ethanol in *Drosophila* using RNAi and pharmacological treatments (Dzitoyeva *et al*., 2003).

The $GABA_A$ receptor is a rapid acting ligand-gated chloride channel, composed of five subunits (Erdozain and Callado, 2014; Stephenson, 1995). Ethanol allosterically potentiates the action of GABA, or any other activator of this receptor such as benzodiazepines or barbiturates, increasing the influx of chloride via $GABA_A$ receptors (Aguayo, 2002). In addition, the effect of ethanol on $GABA_A$ receptors is affected by the composition of the subunits that make up the receptors (Spanagel, 2009) which vary in different brain regions or in cell types in the same region (Grobin, 2000).

The molecular actions by which ethanol may exert its effects on GABAergic activity are by binding directly to the receptor, increasing the presynaptic release of GABA or releasing GABAergic steroids (Lobo and Harris, 2008). Acute ethanol administration has been reported to enhance the function of $GABA_A$ receptors in rats, thereby increasing the chloride influx through the ligand-gated ion channel and increasing GABAergic inhibition (Mihic *et al*., 1997). In contrast, chronic ethanol administration causes a decrease in the sensitivity of $GABA_A$ receptor-mediated responses (Kumar *et al*., 2009). Additionally, chronic ethanol exposure differentially affects the expression of distinct $GABA_A$ receptor mRNA and protein levels in various brain regions and results in CNS excitability during and after withdrawal from exposure to ethanol (Kumar *et al*., 2009).

$GABA_B$ receptors are metabotropic receptors responsible for mediating the slow onset and extended effects of GABA in the brain (Li *et al*., 2005; Most *et al*., 2014). The role of $GABA_B$ receptors in ethanol's effects is still unclear, however, acute ethanol exposure enhances the $GABA_B$-induced synaptic responses in a concentration-dependent manner in rat midbrain dopaminergic neurons (Federici *et al*., 2009). The $GABA_B$ receptor might also play a vital role in controlling the levels of chronic ethanol exposure. For example, the $GABA_B$ agonist, baclofen, suppressed voluntary ethanol consumption in ethanol preferring rats (Vengeliene *et al*., 2008).

1.1.4.3. 5-Hydroxytryptamine-3 ($5HT_3$) receptor

5-Hydroxytryptamine-3 ($5HT_3$), a subtype of serotonin receptor has been identified in several brain regions particularly the hippocampus and it is structurally similar to the $GABA_A$ and nicotinic acetylcholine receptors (nAChR) (Diamond and Gordon, 1997). Ethanol administration has been shown to potentiate the activity of $5\text{-}HT_3$ and the somatodentritic $5\text{-}HT_{1A}$ receptors (Lovinger, 1999). Lynch *et al*., (2011) demonstrated amygdalar 5-HT3 signalling may be involved in controlling ethanol consumption. Additionally, an increase in the extracellular levels of serotonin levels has been reported in the nucleus accumbens after alcohol treatment (Yan, 1999).

Acute ethanol exposure was found to potentiate the effect of $5HT_3$ receptor in oocytes (Harris *et al*., 1995), frontal cortex neurons (Sung *et al*., 2000), ganglion neurons (Lovinger and White, 1991) and human embryonic kidney cells (Lovinger and Zhou, 1994). In contrast, repeated ethanol administration has been associated with a decrease in serotonin functions in the nucleus accumbens in mice by reducing the extracellular concentration of serotonin (Ward *et al*., 2009).

1.1.4.4. Nicotinic acetylcholine receptor (nAChR)

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel with excitatory effects in the mammalian brain (Dick and Agrawal, 2008). It is structurally related to $GABA_A$ receptors (Narahashi *et al*., 1999). nAChRs are widely distributed in the peripheral and CNS (Chatterjee and Bartlett, 2010). They are expressed primarily in the cerebral cortex and some limbic regions with ethanol sensitivities affecting some brain regions more than others (Spanagel, 2009). Ethanol has been reported to act on neuronal nAChRs by enhancing the function of some subtypes and inhibiting the activity of others (Narahashi *et al*., 1999; Davis and de Fiebre, 2006). Neuronal nAChRs have been implicated in acute locomotor response to ethanol effects in both humans and animal models (Kamens and Philips, 2008).

1.1.4.5. Voltage-dependent channels

Voltage-dependent channels including Ca^{2+} channels and K^+ channels, are also known primary targets of ethanol (Diamond and Gordon, 1997; Spanagel, 2009). Acute ethanol administration has been reported to inhibit dihydropyridine-sensitive L-type Ca^{2+} channels (Wand *et al*., 1993). In addition, ethanol opens G protein-activated inwardly rectifying K^+ channels (GIRKs) and has significant implications for inhibitory regulation of neuronal excitability (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). Other voltage-dependent channels including N-, T and P-type channels have also been implicated in the acute effects of ethanol (Walter and Messing, 1999). These channels are activated through depolarization-stimulated calcium influx into neurons thereby increasing their excitability.

1.1.4.6. Other targets of Alcohol

In addition, ethanol affects other neurochemical and endocrine systems. Alcohol acutely activates the mesocorticolimbic dopaminergeric system and upon chronic administration, produces functional alterations of the reward centre (Erdozain and Callado, 2014). The reward centre comprises of the ventral tegmental area and its targets, the nucleus accumbens (NAc), the amygdala. It has been implicated in the rewarding and reinforcing properties of ethanol and other drugs of abuse (Boileau *et al*., 2003; Chao and Nestler, 2004; Koob and Volkow, 2010). Ethanol increases dopamine levels in the nucleus accumbens of humans (Boileau *et al*., 2003). Furthermore, behavioural studies have demonstrated that rats, which are bred to prefer alcohol, produced more dopamine than wild-type rats in an alcohol self-administration study (Weiss *et al*., 1993), supporting the involvement of the dopaminergeric system in alcoholism.

The opioid system has been reported to participate in the reinforcing properties of ethanol in both humans and animals (Arias and Chotro, 2005). The opioid system is associated with several behavioural effects of ethanol such as psychomotor stimulation and sensitization (Font *et al*., 2013). In the endogenous opioid system, ethanol has been reported to affect its receptor subtypes (mu, delta and kappa), as well as the release of the three main classes of endorphins (beta-endorphin, enkephalins and dynorphins) (Gianoulakis, 2009). Acute ethanol stimulates the release of beta-endorphin, enkephalins and dynorphins in humans and animals (Gianoulakis, 1996; Marinelli *et al*., 2006). Furthermore, the administration of opioid antagonists such as naltrexone reduces ethanol intake by decreasing the release of dopamine in the nucleus accumbens (Arias and Chotro, 2005).

Several investigators have suggested that the endocannabinoid system plays an important role during the neurobiology of alcoholism (Al Mansouri *et al*., 2014;

Erdozain and Callado, 2011). Corticotropin releasing factor has also been implicated in ethanol consumption and ethanol addiction processes. In addition, its antagonists have potential as pharmacotherapeutics for alcohol use disorders (Gilpin, 2012a; Zorrilla *et al*., 2013). Similarly, a wide range of evidence suggests a role for neuropeptide Y (NPY) in the regulation of ethanol intake, anxiety-like behaviour and arousal (Gilpin, 2012a; Hayes *et al*., 2012). Finally, receptors such as muscarinic (VanDemark *et al*., 2009) and adenosine (Bulter and Predergast, 2012), are also affected by ethanol.

1.1.5. Pathways associated with alcohol addiction

1.1.5.1. Cyclic adenosine 3', 5'-monophosphate signalling pathway (cAMP pathway)

Ethanol administration has been reported to alter receptor-mediated cAMP signalling transduction in several biological processes and its effects differ according to the expression of certain types of adenylyl cyclases (reviewed in Diamond and Gordon, 1997). cAMP is generated by the enzyme, adenylyl cyclase, and it activates cAMP-dependent protein kinase (PKA). Increased activity of PKA results in the phosphorylation of downstream targets, including cAMP-responsive element binding protein (CREB) (Newton and Messing, 2006). Acute ethanol exposure has been reported to stimulate adenylyl cyclase activity and thus increase cAMP production, while chronic ethanol exposure causes a decrease in receptor-mediated cAMP production (Erdozain and Callado, 2014; Bellen, 1998). Two mechanisms have been proposed for the acute stimulation by ethanol of the cAMP signalling. One mechanism involves the inhibition of the cell surface, type 1 equilibrative nucleoside transporter (ENT1) (one of the main transporters that regulates adenosine in the brain (Dunwiddie and Masino, 2001)), which results in the accumulation of extracellular adenosine and

thus, the stimulation of adenosine A2a receptors coupled to stimulatory G protein (G_s) . The other mechanism, involves the G_s subunit binding directly to and activating specific isoforms of adenylyl cyclases (Gordon and Diamond, 1993).

The importance of the cAMP/PKA dependent pathway has been demonstrated in mice (Wand *et al*., 2001; Yang *et al*., 2003) and *Drosophila melanogaster* (Moore *et al*., 1998). Mice with one disrupted $G\alpha_s$ allele and mice with reduced neuronal PKA activity have decreased alcohol consumption compared with their wild-type littermates (Wand *et al*., 2001). In a follow-up study, mice with $G\alpha_s$ allele did not develop tolerance to the sedative effects of ethanol following subsequent ethanol treatments (Yang *et al*., 2003). Moore *et al*. (1998) provided evidence that cAMP signalling plays a role *in vivo* in the acute response to ethanol in *Drosophila*. They reported that lack of the *amnesiac* gene, a previously identified learning mutant, developed increased sensitivity to alcohol. Further investigation showed that loss of function mutants namely *rutabaga* and *DCO*, which encodes a subunit for cAMP-dependent protein kinase also displayed increased sensitivity to alcohol and were similar to that observed in the *amnesiac* mutants (Moore *et al*., 1998).

Furthermore, ethanol also influences PKA and the downstream effector, cAMP-responsive element binding protein (CREB). Studies have revealed that acute ethanol administration caused translocation of the PKA catalytic subunit to the nucleus (Constantinesu *et al*., 1999) and chronic ethanol administration showed no effect on the levels of the α-subunit of the catalytic domain of PKA (PKA-Cα) in the amygdaloid structure of rats (Pandey *et al*., 2003; Pandey *et al*., 2001) but decreased PKA activity in the NAc (Repunte-Canonigo *et al*., 2007). Similarly, a wide range of evidence suggests that the cAMP-responsive element binding protein (CREB) is also implicated in the neurobiology of alcoholism (Pandey, 2004). Chronic exposure to ethanol produces decreased CREB phosphorylation (Li *et al*., 2003; Misra *et al*., 2001), and remained

decreased during ethanol withdrawal (Li *et al*., 2003; Moonat *et al*., 2010), while no change was observed in total CREB protein levels (Misra *et al*., 2001; Pandey *et al*., 2003). An increase in CREB has been reported after acute ethanol administration (Chao and Nestler, 2004; Pandey, 2004). Other investigators have reported increased expression of phospho-CREB in certain brain regions of mice with $G\alpha_s$ allele following tolerance (Yang *et al*., 2003).

1.1.5.2. Other regulatory pathways

In addition, ethanol affects other molecular signalling pathways, including the mitogen-activated protein kinase (MAP), specifically the extracellular signal regulating kinase (ERK) and the phosphatidylinositol-3-kinase (PI3K)/Akt pathways (reviewed in Ron and Messing, 2013). ERK phosphorylation has been reported to reduce after acute and chronic ethanol exposure in rats and mice (Zhai, 2008) and in postmortem brain of alcoholic subjects (Erdozain *et al*., 2014). The PI3/Akt pathway has been implicated in ethanol's response in *Drosophila melanogaster* (Eddison *et al*., 2011) and rodents (Cozzoli *et al*., 2009).

1.1.6. Regulation of gene expression

Regulation of gene expression at the transcriptional level has been shown to be involved in the development of alcohol addiction (Chao and Nestler, 2004; Nestler, 2012). For instance, acute administration of many drugs of abuse including alcohol, induces transient expression of the *fos* family proteins (Chao and Nestler, 2004). The accumulation of *delta-fosB* has been reported in the nucleus accumbens (NAc) after chronic exposure of drugs of abuse including alcohol (Kelz and Nestler 2000). Additionally, regulation of CREB may further induce other changes that are responsible

for the development of addiction. The cellular pathways associated with CREB, including cAMP signalling pathway, and CREB target genes (NPY and brain-derived neurotrophic factor (BDNF)) can potentially influence down-stream gene expression level (reviewed by Moonat *et al*., 2010). The rate of RNA decay and of other molecules also affects the amount of mRNA molecules available for translation.

Regulation of gene expression via epigenetic mechanisms such as acetylation and methylation of histone proteins and DNA methylation, as well as microRNA (miRNA) have been shown to be associated with alcoholism (Moonat *et al*., 2010; Spanagel, 2009) and to be involved in the establishment of neuronal homeostasis during alcohol exposure (Moonat *et al*., 2010). Several studies have implicated a role for epigenetic mechanisms, especially during induction by chronic ethanol exposure and chromatin remodelling in the development of drug addiction (Renthal and Nestler, 2008). The role of epigenetic mechanisms in alcoholism is underlined by the study of the pharmacotherapeutic effect of histone deacetylases (HDAC) inhibitors, which has recently become an important area of research.

1.2. Experimental models in alcohol research

Experimental models including primates, rodents, fruit flies or yeasts have been widely used by researchers to study alcoholism (Philips, 2002; Tabakoff and Hoffman, 2000). Due to the complexity of alcohol disorders, animal models have been used in an attempt to differentiate the physiological, biochemical, molecular or genetic mechanisms that are believed to be associated with human alcoholism (Tabakoff and Hoffman, 2000). Experimental models allow the researcher to focus on distinct components of the alcohol addiction process, ranging from simple, acute drug responses to complex behaviours such as drug seeking, self-administration and relapse (Kaun *et al*., 2012).

Experimental models enable the investigator to use methods unethical with humans and help simplify complex behaviours. In alcohol research, rodent model organisms, the zebrafish, *Danio rerio*, the nematode, *Caenorhabditis elegans* and the fruit fly, *Drosophila melanogaster* have been successfully employed (Table 1.1).

Table 1.1. Searches on PubMed (http://www.ncbi.nlm.gov/pubmed) with the indicated search word restricted by publication dates

Keyword search	**Number of publications**	
	1994 – 2003	**2004 - 2015**
Alcohol addiction and rodent	1564	1846
Alcohol addiction and primates	11	17
Alcohol addiction and *Danio rerio*	3	28
Alcohol addiction and *Caenorhabditis elegans*	0	23
Alcohol addiction and *Drosophila melanogaster*	11	59

1.2.1. Rodent models

Rodent models such as mice and rats, have been used in the past years to study behaviour relevant to the pathology of alcoholism. Researchers are able to control the intake of alcohol and generate the adaptive changes in the brain associated with the development of tolerance and physical dependence on alcohol (Tabakoff and Hoffman, 2000). Rodents have considerable genetic homology and share the same complex CNS organisation of other mammals (Bennett *et al*., 2006).

Rodent models have been used to investigate the different aspects of alcoholism including, tolerance and withdrawal. Assays such as two-bottle choice model and conditioned place preference using rodents have provided valuable contributions to our understanding of the mechanisms underlying ethanol preference (Green and Grahame, 2008). Pharmacological manipulations (Koob, 2004), selective breeding (Murphy *et al*., 2002) and reverse genetic approaches (Crabbe *et al*., 2006) using rodents have made significant contributions to our understanding of the mechanisms underlying ethanol preference (Devineni and Heberlein, 2010).

Although, rodent models have provided vital insights into the mechanisms underlying ethanol related-behaviour, they are not the ideal model organisms for unbiased, forward genetic approaches to identify novel genes in ethanol preference due to the significant time and expense required for genetic screening (Devineni and Heberlein, 2010; Kaun *et al*., 2012).

1.2.2. *Danio rerio* (Zebrafish)

The small freshwater zebrafish has emerged as a powerful tool for uncovering neural mechanisms of numerous syndromes and disease because of the relative ease of using genetic and molecular tools in this species, coupled with highly neural architecture and the capacity for complex behaviour (Bailey *et al*., 2015b; Lovely *et al*., 2014). Zebrafish responses to alcohol may reveal evolutionarily conserved mechanisms common to vertebrates (Pan *et al*., 2011) and can be immersed into alcohol solutions and for prolonged periods of time if required (Gerlai *et al*., 2006), thus allowing precise and non-invasive drug delivery. Alcohol-induced behaviour during early development of zebrafish has been demonstrated (Lovely *et al*., 2014), including ethanol tolerance and withdrawal (Tran *et al*., 2015). In addition, they have been used to study the teratogenic effects of ethanol (Gerlai *et al*., 2006; Lovely and Eberhat, 2014) and have been proposed as a potential model for fetal alcohol syndrome (Gerlai *et al*., 2008).

1.2.3. *Caenorhabditis elegans* (Nematode)

C. *elegans* model provides a simple system for studying the genetic and molecular effects of ethanol intoxication. They have a short generation time and relatively simple nervous system (Bettinger *et al*., 2004). Ethanol intoxication in *C.elegans* occurs at doses that cause intoxication in other organisms (Davies *et al*., 2003). Genes that alter

ethanol-induced response in the worm also affects ethanol-induced response in rodents (Alaimo *et al*., 2012), thereby suggesting that there are conserved mechanisms for ethanol response between *C.elegans* and mammals (Raabe *et al*., 2014). For instance, the *Clic 4 gene* (a chloride intracellular channel that is involved in the activation of intracellular chloride channel), have been implicated in the behavioural responses to acute responses in *C.elegans*, *Drosophila* and mice (Bhandari *et al*., 2012). The *slo-1 gene* (encoding the BK potassium channel) (Davies et al., 2003) and *NPR-1* (a neuropeptide Y (NPY) receptor-like protein) (Davies et al., 2004) have also been reported in ethanol-induced responses in *C.elegans*. The BK potassium channel can be modulated by G proteins therefore a change in gene expression in G protein could have similar effect to the mutation in potassium channel.

1.2.4. *Drosophila melanogaster* (Fruit fly)

Drosophila melanogaster, also known as the fruit fly, has been intensely studied in biology and has provided crucial insight into cellular, molecular, developmental and disease processes that are conserved in mammals including humans (Devineni and Heberlein 2013; Guarnieri and Heberlein, 2003; Heberlein, 2000; Kaun *et al*., 2012). *Drosophila* has approximately the same number of gene families as those found in mammals (Nichols, 2006). Fruit flies are easy and inexpensive to maintain in the laboratory and have a generation time of approximately two weeks (Devineni and Heberlein, 2013; Guarnieri and Heberlein, 2003). Due to their small size, thousands of genotypes of flies can be maintained in a typical laboratory and they have a rapid life cycle, involving approximately 9 – 10 days at 25^{o}C to develop from egg to mature adult. Also, due to their fertility, hundreds of flies can be obtained from a single female fly (Guarnieri and Heberlein, 2003; Kaun *et al*., 2012).

A major advantage of the *Drosophila* model is the simplicity and scale with which they can be genetically manipulated (Guarnieri and Heberlein, 2003; Heberlein, 2000). Fruit flies are generally found around ripe or fermenting plant material that contains up to 5% ethanol (Guarnieri and Heberlein, 2003; Rodan and Rothenfluh, 2010). The flies are capable of handling the toxic effects of ethanol and they use it as an energy source and as a substrate for lipid synthesis (Kaun *et al*., 2012).

As in mammals, ethanol is oxidised to acetaldehyde in fruit flies by alcohol dehydrogenase (ADH). The ADH in *Drosophila* differs from the mammalian ADH, which is a short chain dehydrogenase and amino acid sequence (Hernandez-Tobias *et al*., 2011; Jornvall *et al*., 1981). The acetaldehyde is subsequently converted to acetate by mitochondrial aldehyde dehydrogenase (ALDH). Both ADH and ALDH functions are crucial to aid resistance to ethanol toxicity (Devineni and Heberlein, 2013). Acetate is converted to acetyl-CoA, which can enter the tricarboxylic cycle or be used as a precursor for the synthesis of saturated fatty acids (Guarnieri and Heberlein, 2003; Sha *et al*., 2014).

Like in mammals, ADH oxidises over 90% ethanol in *Drosophila* and the microsomal and catalase pathways have also been implicated (Guarnieri and Heberlein, 2003). ADH is mostly found in the fat bodies and digestive tract of *Drosophila* larvae and adults (Geer *et al*., 1988). Experimenters have demonstrated a key role for ADH in ethanol metabolism in *Drosophila*. Adult *Drosophila* expressing short-chain ADH emerged shortly after ethanol became available in fermenting fruits (Hernandez-Tobias *et al*., 2011), whereas in the *Drosophila* larvae ADH is up-regulated by the presence of ethanol in food (Mckechnie and Geer, 1984). Thus, *Drosophila* adult and larvae metabolise ethanol differently (Guarnieri and Heberlein, 2003). Additionally, wild-type *Drosophila* is significantly more resistant to the toxic effects of environmental ethanol compared to ADH-deficient strains (David *et al*., 1984). ADH also plays a key role in

Drosophila larvae by catalysing the conversion of ethanol-derived acetaldehyde to acetate (Geer *et al*., 1985).

In previous years, ethanol sensitivity and tolerance have been studied in *Drosophila* using a variety of models/assays. Exposing flies to low concentrations of ethanol elicits locomotor activity in flies (Devineni and Heberlen, 2012, Wolf and Heberlein, 2003), whereas higher concentrations of ethanol lead to decreased activity similar to human intoxication, characterized by loss of postural control and eventually sedation (Corl *et al*., 2009; Devineni and Heberlein, 2012; Moore *et al*., 1998; Singh and Heberlein, 2000).

Loss of postural control in flies was first assayed using the inebriometer, a vertical column containing mesh baffles through which ethanol vapour is delivered (Berger *et al*., 2008; Moore *et al*., 1998). When exposed to ethanol vapour, flies lose postural control and gradually fall from one base to the next. Ethanol sensitivity can therefore be measured as the time required for the flies to reach the bottom of the column. Another assay used to measure ethanol-induced loss of postural control (referred to as sedation) is using line-crossing/locomotion assay, in which one records the number and pattern of grid lines crossed by flies (Singh and Heberlein, 2000).

Ethanol sensitivity has also been assayed by quantifying the behaviour of flies as they recover from intoxication to ethanol (Berger *et al*., 2004), or by using a loss-of- righting reflex (LORR), which involves counting the number of flies that fail to regain upright posture after being knocked over (Corl *et al*., 2009; Devineni *et al*., 2013; Rothenfluh *et al*., 2006) or by quantifying the time it took for half of the ethanol exposed flies to become sedated (Maples and Rothenfluh, 2011).

Recently, more complex assays that are more closely related to mammalian behaviour have been developed. A self-administration assay quantifies ethanol preference in flies

and this preference exhibits several features similar to compulsive alcohol consumption in mammals (Devineni and Heberlein, 2009). To measure whether intoxicating doses of ethanol are rewarding to flies, a conditioned ethanol preference assay was developed (Kaun *et al*., 2011, Shohat-Ophir *et al*., 2012).

Repeated ethanol exposures in *Drosophila* have been reported to induce ethanol tolerance (Scholz *et al*., 2000). Indeed, a single ethanol exposure has the capacity to induce lower sensitivity to a subsequent ethanol exposure (Scholz *et al*., 2000). Several investigators have developed different models to measure ethanol tolerance, which is defined as the acquired resistance to the effects of ethanol after prior ethanol exposure (Berger *et al*., 2004; Scholz *et al*., 2000). Two types of ethanol tolerance have been described in *Drosophila*, rapid and chronic tolerance. Rapid tolerance is caused by brief ethanol exposure whereas chronic tolerance is stimulated by prolonged ethanol exposure (approximately 24 h) that does not produce apparent intoxication (Berger *et al*., 2004). Another type of tolerance which has not yet been characterised in *Drosophila* due to its difficulty in distinguishing from naïve ethanol sensitivity using existing assays, is known as acute functional tolerance. Acute functional tolerance develops within a single intoxicating session (Berger *et al*., 2004, Devineni and Heberlein, 2013).

Several studies have also demonstrated that upon exposure to ethanol, *Drosophila* displays a robust preference for ethanol-containing food with an increase in time and exhibit tolerance with repeated exposure to ethanol (Devineni and Heberlein, 2009; Devineni and Heberlein, 2010; Scholz *et al*., 2000).

Measuring ethanol-induced behaviours in *Drosophila* can be used to understand many different features of the nature of ethanol. Thus, researchers have carried out genetic analyses to identify mutations that alter ethanol sensitivity and development of ethanol tolerance in *Drosophila*. Mutant strains, *tipsy* and *barfly*, obtained by treatment with the

mutagen ethyl methane sulphonate, showed reduced and increased sensitivity to ethanol compared to wild-type flies, respectively (Heberlein *et al*., 2004; Heberlein, 2000).

Another mutant, *cheapdate*, generated by the use of P-element transposable element displayed increased sensitivity to ethanol-induced loss of postural control (Moore *et al*., 1998). Further investigation demonstrated that *cheapdate* flies carry a mutation in a gene called *amnesiac*, which was described initially because of its role in olfactory learning and memory (Quinn *et al*., 1979). In addition to *cheapdate* mutant, other *Drosophila* mutants have been implicated in molecular and cellular processes that mediate ethanol-induced behaviour (Table 1.2).

Table 1.2. Selected mutants invloved in ethanol-induced behaviour in *Drosophila*

Mutants	*Gene*	*Mechanism of action*	*Ethanol-related phenotype*	*Reference*
cheapdate	*amn*	cAMP pathway	Increased motor impairment	Moore *et al*., 1998
sca	*sca*	Notch signalling	Decreased conditioned preference	Kaun *et al*., 2011; LaFerriere *et al*., 2008
hangover	*hang*	Stress pathway	Decreased tolerance	Scholz *et al*., 2005
krasavietz	*kra*	Regulation of translation	Decreased ethanol sensitivity	Berger *et al*., 2008; Devineni and Heberlein, 2009
slowpoke	*slo*	Calcium-activated potassium channel activity	Decreased tolerance	Cowmeadow *et al*., 2005; Cowmeadow *et al*., 2006
happyhour	*hppy*	Inhibits EGFR pathway	Decreased ethanol sedation	Corl *et al*., 2009
arouser	*aru*	EGFR and PI3K/Akt pathways	Increased sedation	Eddison *et al*., 2011
	DopR	Dopamine signalling	Decreased hyperactivity	Kong *et al*., 2010

npf	Neuropeptide (NPF/NPY)	F/Y	Decreased sedation	Thiele *et al*., 1998; Wen *et al*., 2005

This table includes the mutants and genes that have been functionally characterized as regulators of ethanol-induced behaviour. Not all *Drosophila* known mutants are listed but the representative gene for each signalling pathway or general mechanism are listed.

1.3. G proteins

Heterotrimeric guanine nucleotide binding proteins (G proteins) act as intermediaries in the transmembrane signalling pathways that comprises of the G protein-coupled receptor (GPCR), G proteins and downstream effectors (Gilman, 1987; Milligan and Kostenis 2006). GPCRs represent the largest family of transmembrane receptors. They consist of a single subunit with seven transmembrane domains, an extracellular ligand binding site and intracellular G protein binding domain. GPCRs are found in many genomes including humans and they represent a major therapeutic target (Cook, 2010; Johnston and Siderovski, 2007). Binding to a GPCR by their endogenous ligands, facilitates the activation of heterotrimeric G protein (Wettschureck and Offermanns, 2005) which in turn activate different effector molecules such as: ion channels, adenylyl cyclases and phospholipases and phosphodiesterases. Changes in the activity of these effector molecules eventually lead to changes in cellular functions ranging from short term effects like the control of secretion rates, muscle tone or metabolic processes to long term effects like regulation of growth and differentiation (Johnston and Siderovski, 2007; Offermanns, 2003).

1.3.1. G protein signalling

Heterotrimeric G proteins comprise of an α subunit bound to a β and γ subunit (Offermanns, 2003). In the basal state, the guanosine diphosphate (GDP)-bound α

subunit is associated with the βγ heterodimer complex (Elia *et al*., 2005; Offermanns, 2003). Activation of a GPCR by an agonist induces a conformational change within the receptor that is associated with the G protein, thereby resulting in the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit (Johnston and Siderovski, 2007; Offermanns, 2003; Siderovski and Willard, 2005). This leads to the dissociation of the Gα subunit from the βγ heterodimer complex and subsequent modulation of the activity of downstream effectors (Figure 1.4).

G protein signalling is terminated by the hydrolysis of GTP to GDP by the intrinsic GTPase activity of the α subunit. This results in the re-association of the GDP-bound α-subunit and the βγ complex, and the re-association with resting state receptors (Wettschureck and Offermanns 2005; Offermanns, 2003). The GTPase activity of isolated G protein is much lower than those observed in physiological conditions. Several effector proteins interact with the GTP-bound α-subunit and accelerate their GTPase activity, thereby contributing to the deactivation of G protein signalling. A family of proteins called 'regulators of G protein signalling' (RGS proteins) have been identified, which enhances the GTPase activity of Gα subunits (De Vries *et al*., 2000; Ross and Wilkie, 2000) (Figure 1.4).

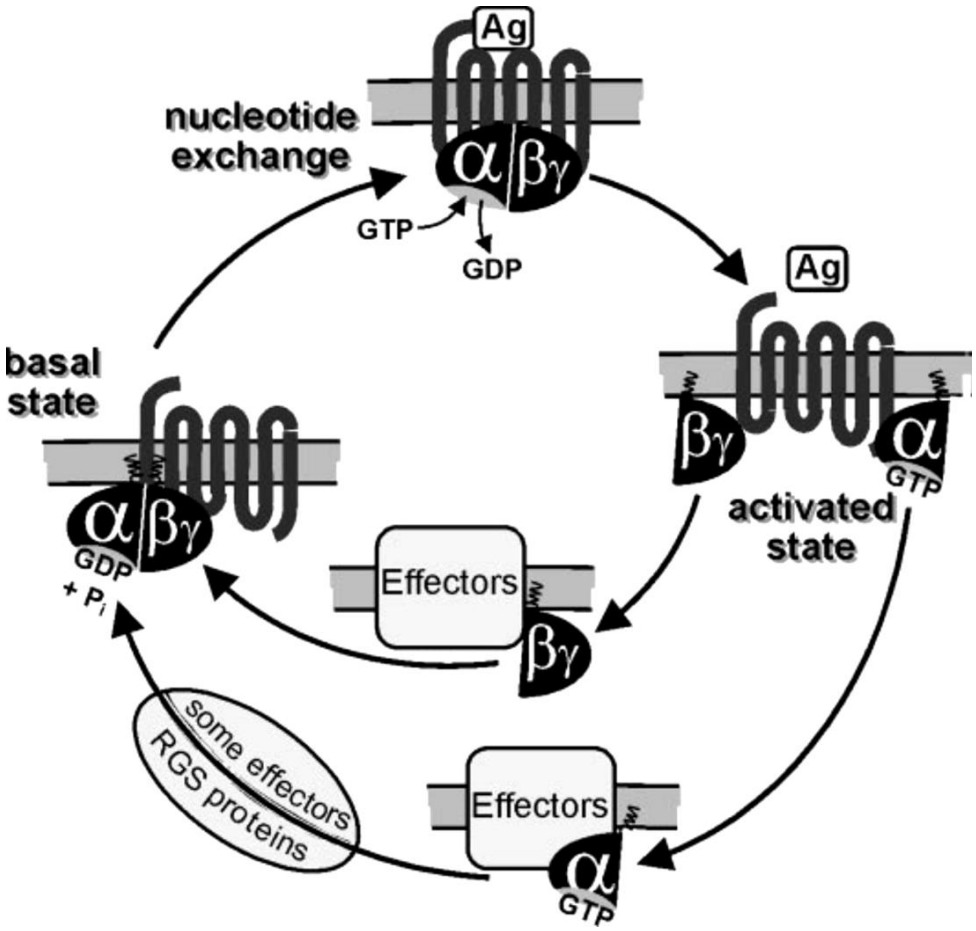

Figure 1.4. Cycle of G protein activity

Agonist (Ag) stimulation of GPCR promotes the release of GDP from the alpha subunit of the heterotrimeric G protein resulting in the formation of GTP-bound Gα. GTP-Gα and Gβγ dissociate and are able to modulate effector functions. The spontaneous hydrolysis of GTP to GDP can be accelerated by various effectors as well as by regulators of G protein signalling (RGS) proteins. GDP-bound Gα then reassociates with Gβγ (With permission from Wettschureck and Offermanns, 2005).

1.3.2. G protein pharmacology

In the human genome, there are sixteen α -subunits divided into four families based on structural and functional homology: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Table 1.3). Although some of the Gα subunits are expressed ubiquitously, others are expressed in a wide variety of tissues and some display a very restricted pattern of expression (Offermanns, 2003). The βγ heterodimer of G proteins in the human genome is made up of five β-

subunits and twelve γ-subunits (Table 1.3). The βγ heterodimer plays an essential role in the regulation of various effector molecules such as the G protein regulated inwardly rectifying K^+ channels (GIRK), particular isoforms of adenylyl cyclase and phospholipase C (Clapham and Neer, 1997).

The activation of a GPCR, usually results in the activation of several signal transduction cascades via Gα subunits as well as through the freed βγ-complex (Wettschureck and Offermanns, 2005). The expression pattern of G proteins activated by a given GPCR determines the cellular and biological response. The activated receptors thereby lead to functionally similar or identical cellular effects usually activating the same G protein subtypes (Wettschureck and Offermanns, 2005). Most GPCRs are able to activate more than one G protein subtype, which leads to the activation of various signalling cascades (Raymond, 1995). Hence, changes in the basal state of G protein subtypes, could have an effect on intracellular signalling pathways by altering the G protein subunit associated with the GPCR and thus affecting the type of effector molecules activated during G protein release (Morris and Malbon, 1999).

G protein subunits show a high degree of interaction specificity between themselves. (Raymond, 1995). Some G proteins are more promiscuous than others, which is especially true for members of the $G\alpha_{q/11}$ subfamily (Kostenis *et al*., 2005; Mody *et al*., 2000). For instance, $G\alpha_{14}$, but most notably $G\alpha_{16}$ and its murine orthologue $G\alpha_{15}$, can link a variety of G_q, G_i and G_s-coupled GPCRs to the phospholipase C (PLC) pathway (Kostenis, 2001; Offermanns and Simon, 1995).

In the *Drosophila* genome, six genes have been described for α-subunits, including G_s, G_f, G_o, G_q, G_i and concertina (cta) (Katanayeva *et al*., 2010) (Table 1.4). Each gene activates different transduction pathways depending on its particular effectors. The pathways for G_s, G_i/G_o and G_q are all well-known and increases cAMP (G_s), decreases

in cAMP (G_i/G_o) and the release of IP3 and diacylglycerol (G_q) (Boto *et al*., 2010) respectively. The signal transduction pathway for G_f (f is for fly) and concertina (cta) are not fully understood. The number of β and γ subunits genes in the *Drosophila* genome is smaller compared with that of humans: only three Gβ genes $G\beta_5$, $G\beta_{76C}$ and $G\beta_{13F}$ (Dolph *et al*., 1994; Yarfitz *et al*., 1988) and two genes encode for the Gγ subunits, $G\gamma_1$ and $G\gamma_{30A}$ (Ray and Ganguly, 1992; Schulz *et al*., 1999) (Table 1.4).

Table 1.3. Human G proteins

Name	Subtypes	Expression	Effectors	Roles
α-subunits				
$G\alpha_s$	$G\alpha_s$, $G\alpha s_{XL}$ and $G\alpha_{olf}$	Ubiquitous, neuroendocrine and olfactory epithelium, brain respectively	AC	G_s and G_{olf} are the two main members of the Gαs family. Gs act on the signalling pathway through GPCR to elevate cAMP .
$G\alpha_{i/o}$	$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_{gust}$, $G\alpha_{t\text{-}r}$, $G\alpha_{t\text{-}c}$	Widely distributed, ubiquitous, widely, neuronal, platelets, taste cells, retinal rods and retinal cones respectively	AC, PDE, GIRK	They mediate GPCR dependent inhibition of AC. Most of the effects of G_o activation are mediated by βγ-subunit. G_o is associated with Ca^{2+} channels and GIRK.
$G\alpha_{q/11}$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15/16}$	Ubiquitous, almost ubiquitous, kidney& lung, hematopoietic cells respectively	PLC β-isoforms	Gαq & Gα11 are involved in the coupling of receptors in a pertussis toxin insensitive manner to PLC β-isoforms.
$G\alpha_{12/13}$	$G\alpha_{12}$, $G\alpha_{13}$	Both Ubiquitous	RhoGEF	They are activated by receptor-coupling $G_{q/11}$.
β-subunits				
β-subunits	B, β2, β3, β4, β5	Widely& retinal rods, widely distributed, widely & retinal cones, widely and mainly brain respectively	Isoforms of AC, PLC, GRK, receptor kinases, GIRK, PI-3-K	The Gβγ subunits are involved in the activation of K^+ channels in the heart and the inhibition of ca^{2+}/calmodulin-stimulated AC activity in the brain. They are also involved in the stimulation of PLC activity in the brain.
γ-subunits				
γ-subunits	Γ1, γrod, γ14, γcone, γ2, γ6, γ3, γ4, γ5, γ7, γ8, γ9, γ10, γ11, γ12, γ13	Mostly widely distributed, retinal rods and brain		

AC: adenylyl cyclase; GPCR: G protein-coupled receptor; PDE: phosphodiesterase; GIRK: G protein regulated inward rectifying potassium; PLC: phospholipase C; GRK: G protein regulated kinase; PI-3K: phosphatidylinositol-3-kinase; RhoGEF: Rho guanosine nucleotide exchange factor (Adapted from Wettschureck and Offermanns 2005; Offermanns, 2003)

Table 1.4. *Drosophila* G proteins

Name	Family	Human orthology	Roles
α-subunits			
$G\alpha_s$	$G\alpha_s$	$G\alpha_s$	Plays a role in larval growth (Wolfgang *et al*., 2001)
$G\alpha_i$	$G\alpha_{i/o}$	$G\alpha_i$	Involved in the formation of blood-brain barrier (Schwabe *et al*., 2005)
$G\alpha_o$	$G\alpha_{i/o}$	$G\alpha_o$	Involved in feeding behaviour (Fitch *et al*., 1993) and learning and memory (Ferris *et al*., 2006)
$G\alpha_q$	$G\alpha_{q/11}$	$G\alpha_q$	Plays a role in phototransduction (Scott *et al*., 1995) and olfaction (Kalidas and Smith, 2002)
$G\alpha_f$	$G\alpha_f$	none	The signal transduction pathway is unknown. Expressed in embryonic, larvae and early pupae stages of development (Quan *et al*., 1993).
concertina (cta)	$G\alpha_{12/13}$		Its signal transduction is not fully understood but plays a role in *Drosophila* gastrulation (Peter and Rogers, 2013).
β-subunits			
$G\beta_5$			Expressed in olfactory receptor organs (Boto *et al*., 2010)
$G\beta_{13F}$			Plays a role in the control of asymmetric cell divisions in the neuroblast and sensory organ lineages (Schaefer *et al*., 2001).
$G\beta_{76C}$			Expressed in the eye of *Drosophila*. Its localisation suggests a role in phototransduction (Dolph *et al*., 1994).
γ-subunits			
$G\gamma_1$			Phototransduction (Schulz *et al*., 1999)
$G\gamma_{30A}$			Phototransduction (Schulz *et al*., 1999)

1.3.3. G proteins and ethanol-mediated behaviours

Chronic administration of ethanol causes profound, long-lasting behavioural and biochemical changes, which are the basis for the phenomena of ethanol tolerance, dependence and abstinence syndrome after ethanol withdrawal (Moonat *et al*., 2010). These prolonged changes, which are induced by alterations in receptor-activated intracellular signalling cascades affecting the nucleus, results in changes in the expression levels of immediate early-genes, neurotransmitter transporter and heterotrimeric G protein-coupled receptors (GPCRs) (Harlan and Garcia, 1998; Kitanaka *et al*., 2008). Several studies have revealed that chronic administration of abused drugs such as ethanol can alter signalling pathways such as cAMP pathways (Nestler and Aghajanian, 1997).

Studying the expression levels of G protein subunits can identify and unravel specific intracellular effectors associated with ethanol exposure. Previous investigators have implicated chronic ethanol administration to alter G protein subunits associated with the cAMP pathway. A reduction in the $G\alpha_s$ subunit (Mochly-Rosen *et al*., 1988) and/or increase in $G\alpha_i$ subunit (Charness *et al*., 1988) of the heterotrimer G proteins appear to account for heterologous desensitization of cAMP signalling caused by chronic ethanol exposure in neuroblastoma cells (Diamond and Gordon, 1997). In another study, a reduction in the G_s subunit was also observed in erythrocyte membranes from alcoholics (Nakamura, 1994). Another study reported, no change was detected in some Gα and Gβ subunits following acute treatment to ethanol in rat's cortical and cerebellar membranes using immunoblotting assay (Hatta *et al*., 1994; Wand *et al*., 1993). Other investigators reported an increase in the protein levels of one $G\alpha_o$ isoform in rats cerebellum after chronically ingested ethanol (Guillen *et al*., 2003). Some of the variations within these studies, might be due to the regimes of ethanol administration and the type of assay utilised.

One of the molecular targets of ethanol is the G-protein-coupled inwardly rectifying potassium channel (GIRK), which is coupled to G protein subunits, G_i and G_o. Chronic treatments of rats with ethanol showed decreased guanosine nucleotide binding protein, beta polypeptide (GNB1) gene in the hippocampus, so that chronic ethanol intake may reduce the availability of Gβγ dimers, resulting in reduced GIRK activation (Saito *et al*., 2002).

A wide array of studies have demonstrated that abused drugs such as opiates (Chetswang *et al*., 1999; Kaewsuk *et al*., 2001; Nalepa *et al*., 2007; Narita *et al*., 2001; Nestler *et al*., 1989; Zelek-Molik *et al*., 2012), cocaine (Carrasco *et al*., 2003; Perrine *et al*., 2005) and barbiturates (Kitanaka *et al*., 2008) can alter the level of G protein gene expression in rat brains. Though, there are only a few reported studies that investigate the effect of ethanol-induced behaviour on G protein subunits, none have employed adaptive changes to ethanol treatment. To this author's best knowledge, this is the first report demonstrating G protein gene expression changes using real-time polymerase chain reaction.

1.4. Overall aim

The overall aim of the work described in this thesis is to better understand if ethanol can cause changes in the level of G protein gene expression in *Drosophila* that correlate with ethanol-induced behavioural changes. The model, *Drosophila* was selected due to its simplicity and accessibility to genetic, molecular and behavioural analyses (Bainton *et al*., 2005).

1.4.1. Research rationale

The justification of these investigations is given that G proteins can be promiscuous in their binding to receptors and given that different G proteins activate different effector molecules, a change in the relative complement of G proteins could result in different G proteins being associated with the neurotransmitter receptors. Thus a different response could occur following the same stimuli or absence of stimuli in an organism previously exposed to an addictive drug. Hence, it is important to establish whether the expression of specific G proteins is affected, how such changes occur and what the consequences are.

1.4.2. Objectives

- To develop a suitable model of ethanol-induced behaviour in which G protein gene expression changes can be measured.
- To identify any G protein subtypes which might be up-regulated or down-regulated following ethanol-induced behaviour.
- To evaluate the significance of any identified changes in ethanol-induced G protein gene expression by utilizing *Drosophila* mutants.

1.4.3. Research hypothesis

Ethanol administration can alter G protein gene expression levels in the head of *Drosophila* and such changes can explain, at least partially, the development of alcohol addiction in flies and possibly also in humans.

1.4.4. Original contribution to knowledge

To date, there has been no reported research on the effect of alcohol on G protein gene expression in *Drosophila*. The original contribution of this research to knowledge is the demonstration that in *Drosophila* alcohol induces changes in G protein mRNA level. These changes could affect the signal transduction pathways and contribute to the molecular mechanism underlying drug addiction. If these changes in gene expression are maintained, they could play a role in drug related conditions such as withdrawal and relapse.

Chapter 2: Materials and methods

2.1. *Drosophila* handling and strains

2.1.1. *Drosophila* handling and husbandry

Drosophila melanogaster, were maintained either at 25ºC (their life cycle is approximately 9-10 days) during experiments or at 18ºC (their life cycle is about 20 days) for long-term storage of stocks according to previously established protocols (Dahmann, 2008). At both temperatures, the flies were kept in a 12h light/dark cycle incubator with 60% relative humidity. The flies were grown on a Ready-mix *Drosophila* dried food prepared with water in equal amounts and sprinkled with a small amount of dry yeast (Philip Harris Education, UK) in plastic fly vials measuring 25 x 95mm optical density (OD) x height (H) (Dominique Dustcher, FR) or in bottles measuring 240 x 350mm diameter to expand population, stoppered with cotton-like plug or cotton wool.

2.1.2. *Drosophila* Strains

The strains of *Drosophila* used in this study include wild-type *Canton-S*, *Drosophila* mutants for G proteins and dopamine 1-like receptor type 2 (Table 2.1). All fly strains were obtained from the *Drosophila* stock centre Bloomington (Bloomington, IN, US).

Table 2.1. The fly strains used in the study

Fly line	Bloomington stock number	Notes
Wild-type (*Canton-S*)		Wild-type strain of *Drosophila*, which has been maintained in the laboratory originally from Philip Harris Education UK. Used in most behavioural assays and as a control
G_q protein	30736	Mutation of the G protein subunit, G_q on the 2nd chromosome (Kain *et al*., 2008)
G_i protein	17672	Mutation of the G protein subunit, G_i on the 3rd chromosome (Bellen *et al*., 2004)
Dopamine receptor	24743	Mutation of the dopamine 1-like receptor type 2 on the 3rd chromosome (Metaxakis *et al*., 2005)

2.1.3. *Drosophila* collection

All flies used in experiments were collected at 25^{o}C between 1-3 days old so as to reduce age-related behavioural effects. All assay-specific experiments started at the same time of the day (in the morning) to reduce errors due to variation in results that may be caused by circadian rhythms (Van der Linde and Lyons, 2011).

The flies used in this study were females, (unless otherwise stated) this is taking into account the need to remove any effect due to possible sexual dimorphism and reduce size discrepancy between individual. Flies were sorted by gender under the microscope by anaesthetization with carbon dioxide (CO_2) at least 24h prior to the start of the experiments to allow recovery. Males are generally smaller than females and have a rounder abdomen that is dark dorsally compared with the striped tip of the female abdomen (Dahmann, 2008).

For all experimental crosses, it was important to use virgin females. This is because mated females store sperm in the ventral receptacle and spermatheca. This stored sperm enables female flies to lay up to 100 eggs per day in ideal conditions (Roberts, 1998).

Because male and female flies develop from the same vial or bottle, brother-sister mating can occur, thereby confusing the results of the crosses. Using virgin females therefore ensures crosses produce progeny at expected Mendelian ratios. Female flies will not mate within the first eight hours of eclosion at 25ºC. The virgin females were collected when a vial or bottle of all adult flies was emptied in the morning and collected within eight hours and stored in a fresh vial. The virgin female flies can also be identified by the light colour of their cuticle and by a dark spot that can be easily seen through the white abdomen (Greenspan, 2004).

2.2. Behavioural assays

In order to measure ethanol-induced behaviour in *Drosophila*, different behavioural assays were considered and tested. One was the ethanol preference assay that is used to measure ethanol self-administration in flies using the capillary feeder assay (CAFE) (Devineni and Heberlein, 2009), the other assay considered was the assay previously described by Maples and Rothenfluh, 2011, which measures ethanol sensitivity and tolerance. The disadvantage of using the preference assay is that it is dependent on a number of different variables such as length of pre-exposure, concentration of ethanol used in the pre-exposure and in the testing. The method described by Maples and Rothenfluh, 2011 was chosen because of its ability to measure ethanol sensitivity and tolerance in flies and also due to its simplicity and reproducibility.

2.2.1. Ethanol sensitivity assay

The ethanol sensitivity assay previously described (Maples and Rothenfluh, 2011), was modified and used to test for ethanol sensitivity in *Drosophila*. For this assay, eight female flies were used for each group within an experiment. The flies were collected

under CO_2 anaesthesia, stored in new food vials without yeast and allowed to recover 24 h before testing, so as to avoid any effects of the CO_2 on ethanol sedation. Exposures to ethanol were performed using empty plastic vials (25 x 95mm) that had cotton-like plug pushed to the bottom of the vial to make a smooth surface, so that the flies would not get stuck at the bottom of the vial. Flies were then exposed to 500µl of 100% ethanol placed on the bottom of a new cotton-like plug making sure the ethanol faces the bottom of the exposure chamber and not the wall of the vial (Figure 2.1).

During exposure, the exposure chamber was tapped every minute to startle the flies and observed for 10s. The number of stationary flies was recorded for each minute. Flies were stationary if they were unable to turn over, remain in one position or rapidly vibrate their wings throughout the ten-second-observation period. Flies were not stationary if they could turn over or walk up and down the vial. The time required for four of the eight ethanol-exposed flies to remain stationary is defined as the time to 50% sedation (ST50). For the gene expression studies, flies were exposed for a duration of 30 min in order to avoid lethality that can occur with longer exposures of ethanol.

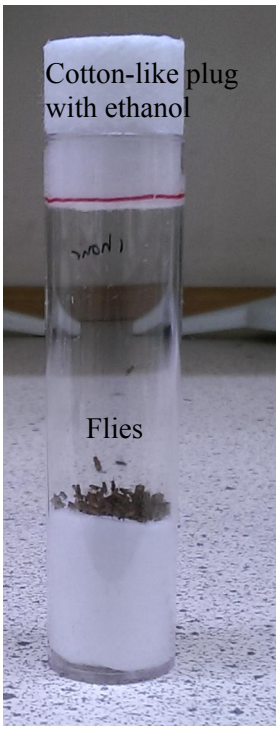

Figure 2.1. Image showing the apparatus used in behavioural assays

Flies were placed in a plastic vial that had cotton-like plug pushed to the bottom to make a smooth surface. Flies were then exposed to 500µl of 100% ethanol placed on the bottom of a cotton-like plug, making sure the ethanol faces the bottom of the plastic vial. The red line indicates the surface on which ethanol was added. The number of stationary flies was recorded every minute for 10s.

2.2.2. Tolerance assay

The tolerance assay was carried out as described in (Maples and Rothenfluh, 2011) with slight modifications. Age-matched female flies (1-3 days old) were pooled from the same culture. The flies were divided into four groups of eight flies each (one, two, three and four exposures) that were placed on one of four exposure chambers.

In the four exposure-group, ethanol tolerance was determined in the ethanol sensitivity assay described in Section 2.2.1. Flies were exposed to ethanol and the time to 50% sedation was recorded. The flies were then allowed to recover 24h in food vials at 25^{o}C,

subjected to a second and third exposure to ethanol, allowed 24h recovery between each exposures and then the fourth exposure in the ethanol sensitivity assay. This is repeated for the other groups of flies (three, two and one), allowing 24h recovery between each treatments. Ethanol tolerance was also determined in the four, three, two and one-exposure groups as described above (Figure 2.2). The development of tolerance was measured as an increase in ST50 and reduced effect of ethanol after prior ethanol treatment as similarly reported (Awofala, 2011; Chan *et al*., 2014; Maples and Rothenfluh, 2011).

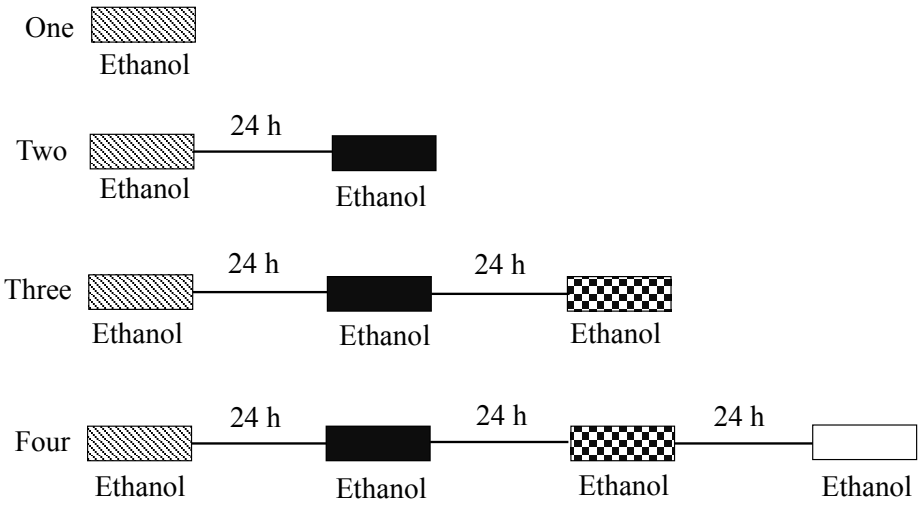

Figure 2.2. Schematic of the four ethanol exposed groups used to determine when flies acquire tolerance to the sedating effects of ethanol.

Flies were exposed to 500µl of 100% ethanol one, two, three and four days, allowing 24h recovery period.

2.3. Subpopulation of wild-type *Drosophila* (early and late responders to ethanol)

2.3.1. Separation of early and late responders

Twenty males and twenty virgin female flies (1-3 day old) were collected separately from the same culture of wild-type *Drosophila*. Using an exposure chamber (Figure 2.3) manufactured in the laboratory for the collection of flies, the flies were exposed separately to 500µl of 100% ethanol placed on a cotton wool attached to a container. The flies were collected into a hole created on a petri dish using a paint brush when observed for 10s every min to determine when they became stationary. The first 20% (4 flies) of male and female flies that remained in one position or vibrate their wings were collected and identified as early responders to ethanol while the last 20% (4 flies) of male and female flies were collected and identified as late responders to alcohol (Figure 2.4).

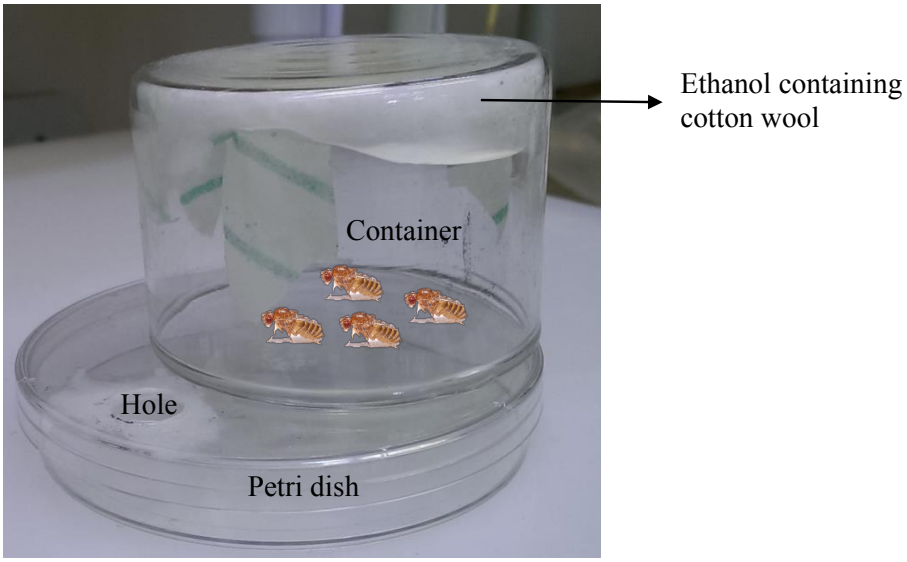

Figure 2.3. Image showing the apparatus used for the separation of early and late responders.

Flies exposed in the chamber were recorded every minute for 10s and collected using a paint brush into the petri dish through the hole.

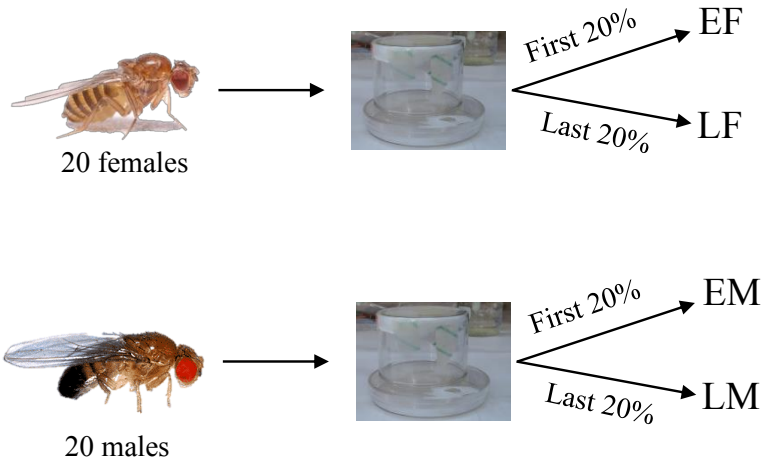

Figure 2.4. Scheme showing how male and female flies were separated.

Flies were separated using the exposure chamber in Figure 2.3 into EF: early female, LF: late female, EM: early male, LM: late male.

2.3.2. Experimental cross of early and late responders

Following the segregation of early and late responders, the early male (EM) and early female (EF) flies were placed in a fresh bottle of *Drosophila* food and allowed to breed. The late male (LM) and late female (LF) flies were placed in another bottle of fresh *Drosophila* food and allowed to breed. This was identified as the parental generation of early and late responders (Figure 2.5).

After breeding, the bottles were cleared so as to collect virgin female flies from the early responders (EM X EF) and late responders (LM X LF). The progeny (males and virgin females) of the parental generation of early responders (EM X EF) were collected and separated based on their response to ethanol as described above. The first 20% of male and female flies were bred in a fresh bottle of food (F1: EM X EF). The progeny (males and virgin females) of the parental generation of late responders (LM X LF) were collected and separated. The first 20% of male and female flies were bred in a fresh bottle of food (F1: LM X LF). This was identified as the first generation of offspring (F1 generation). This process was repeated to identify the second generation of offspring (F2 generation).

Tolerance was measured in the parental generation of early and late responders to ethanol. Eight female early and late responders were collected the day before exposure and exposed to ethanol consecutively for three days as described in Section 2.2.2.

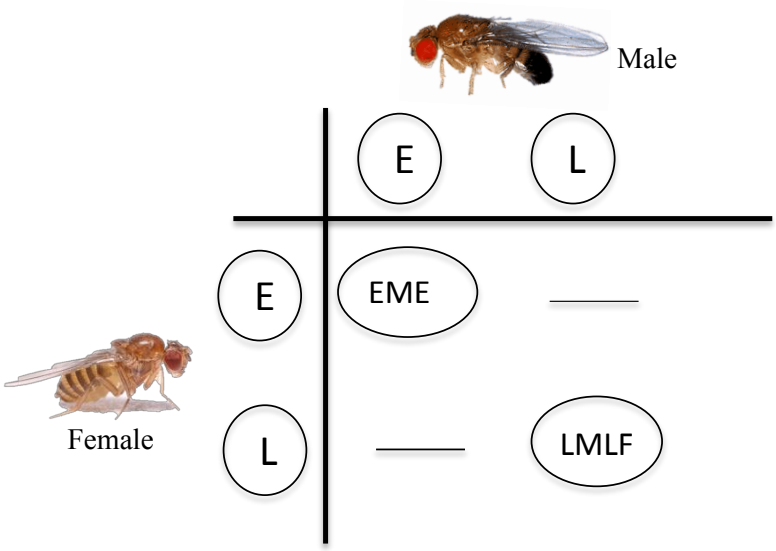

Figure 2.5. Experimental cross of early and late responders.

The experimental cross between early (E) and late (L) male (M) and female (F) responders.

2.4. Enzyme assay - Alcohol dehydrogenase assay (ADH)

2.4.1. Isolation of protein from fly

Five whole early, late and wild-type female flies were collected separately in an Eppendorf tube and frozen in liquid nitrogen for 10min. Homogenates were prepared by manual grinding of the flies in sterilized tubes with plastic pestle. Homogenization was carried out in 50µl of ice-cold protein extraction (PE) buffer (0.1M phosphate buffer, pH 7.4, 0.5M EDTA). Homogenates were then centrifuged at 10,000g for 15min at 4^{o}C. The supernatant was isolated and stored in -20^{o}C until used.

2.4.2. ADH assay

ADH activity was assayed as previously described (Pfeller *et al*., 2005) between the parental generation of early and late responders using wild-type flies as a control. 10µl of supernatant was added to 100µl of assay medium containing 0.052M Tris-HCl (pH 8.0), 5.1mM NAD^+ and 0.20M 2-propanol as substrate in a 96 well microtitre plate (Thermo Scientific, UK). The absorbance at λ= 340nm was measured every 10s for 5 min using a Thermo Multiskan plate reader (Thermo, US). Reactions were carried out in triplicate. A molar extinction coefficient for NADH of 6.220 mM^{-1} cm^{-1} was used to estimate NADH production. The protein content was determined by the Bradford assay (See Section 2.4.3.)

ADH activity was calculated as follows:

$$\text{ADH activity} = \frac{\Delta A340/\text{min} \times \text{total volume}}{\text{Coefficient of NADH} \times \text{volume of extract} \times \text{protein concentration}}$$

2.4.3. Protein assay (Bradford assay)

Protein samples extracted from the flies (Section 2.4.1) were performed using the method based on Bradford (Bradford, 1976). Using bovine serum albumin (BSA) as a standard, 1mg/ml was prepared by diluting 5mg of BSA and 5ml of distilled water. The stock (1mg/ml) was used to make different concentrations of BSA standard (0.05mg/ml, 0.1mg/ml, 0.2mg/ml, 0.3mg/ml, 0.4mg/ml, and 0.5mg/ml). BSA standards were quantified by mixing 10µl of standards with 200µl of Bradford's reagent (1:5 dilution) (BioRad, Inc, US) in a 96 well microtitre plate (Thermo Scientific, UK) and incubating at room temperature for 5min and then measuring the absorbance at λ= 595nm using a Thermo Multiskan plate reader (Thermo, USA). Assays were performed in triplicate

and a standard curve was plotted. 10µl of protein samples were added to 200µl of diluted Bradford reagent. Absorbance at λ= 595nm was measured and the amount of total soluble protein determined from the standard curve.

2.5. Polymerase Chain Reaction (PCR)

2.5.1. Total RNA preparation

Five whole flies were placed in a 1.5ml Eppendorf tube and frozen in liquid nitrogen for 10min. The fly heads were isolated by 10s vortex decapitation and selected under the microscope. The five pooled heads were then preserved in 10µl of RNA Later (Qiagen Inc., US) until used. RNA Later solution is used for immediate stabilization of the RNA. Homogenates were prepared by manual grinding of fly heads in a fresh sterilized 1.5ml Eppendorf tubes with a plastic pestle. Total RNA was isolated from fly heads using an RNeasy Plus Micro Kit (Qiagen Inc., US) according to the manufacturer's instructions. Briefly, total RNA was eluted in 14µl of RNase free water (Qiagen Inc., US). The quality and quantity of RNA were assessed by placing 1µl of RNA sample onto a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, US). Samples with a λ= 260/280nm ratio between 1.8 and 2.1 were used for experiments. RNA was stored at -80ºC until used.

2.5.2. Sequence analysis and design of primers

The DNA sequences for G protein genes in the *Drosophila* genome were obtained from FlyBase (www.flybase.org) and/or National Centre for Biotechnology Information Databases (NCBI). The six genes that encode for Gα subunits (G_i, G_q, G_f, G_o, G_s and concertina (cta)) all had multiple transcript variants due to alternative splicing of mRNA except G_i and G_f genes. The three genes that encode for the Gβ subunits ($G\beta_{13F}$, $G\beta_{76C}$

and $G\beta_5$), possess one transcript variant except $G\beta_{13F}$ which has 6 transcript variants. Both Gγ genes, ($G\gamma_{30A}$ and $G\gamma_1$) possess multiple transcript variants. The G protein genes with multiple transcript variants were first aligned in individual gene using a free online tool through the European Bioinformatics Institute (EBI) known as CLUSTALW2 (www.ebi.ac.uk/tools/ms/clustalW2). This alignment enables the identification of regions that are similar within the transcripts of each gene (See appendix 2.1).

Oligonucleotide primer pairs of all G protein subunits were designed using primer-BLAST (NCBI). Primers were designed to span an exon-exon junction to avoid amplifying contaminating genomic DNA, with the exception of $G\beta_5$ whose gene lacks introns. Primer pairs were verified by a BLAST (Basic Local Alignment Search Tool) search to be specific to each G protein and a predicted amplicon size was obtained (Table 2.2). The sequence for primers for the housekeeping gene, β- actin was selected from a previous study (Ponton *et al*., 2011). Primers were purchased from Eurofins Scientific (UK). Primers were re-suspended in RNase free water (Qiagen Inc., US) to a concentration of 100μM. The primers were diluted to a working concentration of 10μM and stored at -20^{o}C until used.

Table 2.2. Primer sequence and product length for all G protein genes (Gα, Gβ and Gγ) and house-keeping genes (β-actin)

G Proteins	Sequences	Amplicon length(bp)	Annealing temperature (°C)
Gα genes			
Gi (CG10060: G protein αi subunit 65A)	F: CGCGCAATGGGACGCCTGAA	106	55
	R: GCAGCAGGATGCCCTCGTCG		
Gq (CG17759: G protein α 49B)	F: CAGCAGCACGCGAAAGCGTC	114	65
	R: GTCCCGGCGCAACTGCTTCT		
Gf (CG12232: G protein α 73B)	F: CATGGGTGGTGGCGAACAGCAG	120	65
	R: CTGCACGAGATCAGGAACAATACGG		
Go (CG2204: G protein oα 47A)	F: AACGCCTCTGGCAGGACGCC	115	
	R: TCCTTGGCGCCTAACCGATCCA		65
Gs (CG2835: G protein sα 60A)	F: AGCAGGATATTCTTCGGTGCCGT	118	55
	R: TTCCTACGCTCGTCCCGCTG		
cta (CG17678: Concertina)	F: TTCGTTGATGTTGGTGGACAGCG		
	R: TCCGCAAGAACTTGATCGAACTCCG	119	65
Gβ genes			
Gβ13F (CG10545: G protein β-subunit 13F)	F: CGTGGGTGATGACCTGTGCGT	120	59
	R: CACGGGACACCCGGACGTTG		
			55
Gβ76C (CG8770: G protein β-subunit 76C)	F: ACCATCCCAGTGGCTTCGGGT	116	

	R: GCCAGTGTTCTTCTGGGGCGG		
Gβ5 (CG10763: Gβ5)	F: TCTGGGACATGCGCTCTGGTCA	117	59
	R: TGCTGTCATCCGATCCAGTGGC		
Gγ genes			
Gγ30A (CG3694: G protein γ30A)	F: TCTGGTGCCGGTAGAGATGCAG	120	65
	R: TGAATGCTCCGCTTGCCCCC		
Gγ1 (CG8261: G protein γ1)	F: CGTTGCCGAGGAGTCAGCGA	120	65
	R: TCCAGGTGGCGTTGATACTGGT		
Housekeeping gene			
β actin (CG12051: Actin42A)	F: GCGTCGGTCAATTCAATCTT	138	55
	R: AAGCTGCAACCTCTTCGTCA		

2.5.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to test for the presence of G protein mRNA in the head of flies. The primers used for amplifying the specific PCR products are listed in Table 2.2. Each RT-PCR reaction was carried out using SuperScript ® III One-Step RT-PCR system with Platinum® *Taq* DNA polymerase (Invitrogen, UK). In all cases, the amplification reaction was carried out in 25µl total volume consisting of 1µg of RNA, 12.5µl of 2X reaction mix (a buffer containing 0.4mM of each dNTP, 3.2mM $MgSO_4$), 200nM of forward primer and 200nM of reverse primer, 1µl of SuperScript ® III RT/ Platinum® *Taq* Mix and 3.7µl of Nuclease free water (Qiagen Inc., US). PCR was carried out on a BioRad My Cycler, thermal Cycler™ (BioRad, Inc, US) as follows: cDNA synthesis at 60ºC for 30min, initial denaturation at 94ºC for 2min and 40 cycles of denaturation temperature at 94ºC for 15s, annealing temperature between 55ºC and 67ºC for 30s and extension temperature of 68ºC for 1min with a final extension time of 5 min.

2.5.4. Agarose gel electrophoresis of DNA

After amplification, 10µl of each of the PCR products were mixed with 2µl of 6X loading dye (50% (v/v) glycerol, 0.1% (w/v) bromophenol blue, distilled water). 1.5% agarose gels were prepared in conical flasks with agarose (Sigma, US) in 1X Tris/borate/EDTA (TBE) buffer containing 0.089M Tris-base, 0.089M boric acid and 0.002M EDTA with 15µl Safe view, Nucleic acid stain (NBS Biologicals Ltd, UK) and were placed horizontally in a SCIE-PLAS gel electrophoresis tank (BioRad, Inc, USA), containing enough TBE buffer to cover the gel. PCR products were loaded onto the gel. A marker, GeneRuler™ 100bp Plus DNA Ladder ranging from 100bp to 3000bp (Fermentas, Thermo Scientific, UK) was also electrophoresed to estimate the band size

of samples. The agarose gel was visualized using GeneSnap from Syngene (Syngene, UK).

2.5.5. Quantitative Real-time polymerase chain reaction (qRT-PCR)

Quantitative Real-time PCR was carried out by analysis of each RNA sample in triplicate using EXPRESS one step SYBR GreenER kit (Invitrogen Corporation, US) on a Stratagene Mx3000p^{TM} Real-Time PCR System (Stratagene, US) according to the manufacturer's instructions. The primers used for qRT-PCR are listed in Table 2.2. The housekeeping gene β-actin was used to allow relative quantification of RNA and normalisation of each gene. qRT-PCR efficiency was determined for each gene with the slope of a linear regression model (Pfaffl, 2001). Relative standard curves for the genes were generated with serial dilutions of RNA prepared from fly heads (i.e. 1:10, 1:100, 1:1000, and 1:10000). The Corresponding qRT-PCR efficiencies (E) were calculated according to the equation: $E = (10^{(-1/slope - 1)} \times 100$ (Fraga *et al.*, 2008).

Each reaction mixture contained the following: 1µg of diluted RNA, 200nM of forward and reverse primer, 10µl of EXPRESS SYBR GreenER™ qPCR Supermix with premixed ROX, 0.5µl of EXPRESS Superscript mix, made up to 20µl with nuclease free water (Qiagen Inc, US) in a 96-well plate (Thermo Scientific, UK). qRT-PCR was performed under the following sequential conditions according to manufacturer's protocol: cDNA synthesis at 50^{o}C for 5min, initial denaturation at 95^{o}C for 2min, followed by 40 cycles of denaturation at 95^{o}C for 15s and annealing at their respective temperatures and extension at 60^{o}C for 1min. In each experiment, a melting curve cycle was performed according to the manufacturer's programme to check the melting temperature of the products produced to ensure the product was the size expected and not the result of primer-dimers (Appendix 2.2).

2.5.6. Nucleotide sequencing

Nucleotide sequencing was used to confirm the validity of the primers of interest. For economic reasons, both in time and money, sequencing was carried out by Eurofins Scientific (UK). RT-PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc, US). 2ng cDNA samples and primers at 10μM were sent to the company. The concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, US).

2.6. Western blot

2.6.1. Buffers

Tris-EDTA (TE) Buffer: 20mM Tris base**,** pH8, and 1mM ethylenediaminetetraacetic acid (EDTA).

Running Buffer: 25mM Tris base and 250mM glycine at pH8.3 with 0.1% (w/v) sodium dodecyl sulfate (SDS)

Transfer Buffer: 25mM Tris base and 192mM glycine at pH 8.3 with 20% (v/v) methanol

Washing Buffer: 20mM Tris base and 500mM sodium chloride at pH7.5 with 0.1% Tween-20. Also known as Tris buffered saline-Tween (TBST)

Blocking Buffer: 5% non-fat milk in TBST and 5% bovine serum albumin (BSA) in TBST

All chemicals are from Sigma (US), unless indicated.

2.6.2. Antibodies

Primary antibodies used were rabbit polyclonal anti-$G\alpha_{q/11}$ at a dilution of 1:500; goat polyclonal anti-$G\alpha_{i/o/t/z}$ at a dilution of 1:500 (Santa Cruz Biotech. Inc. US) and anti-actin loading control at dilution of 1:1000 (Abcam, UK). Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG (Cayman Chemical Company, UK) and peroxidase conjugated rabbit anti-goat IgG produced from mouse (Boster Biological Tech. Ltd, US). The secondary antibodies were used at 1:1000 and 1:5000 dilutions respectively.

2.6.3. Isolation of protein from fly heads

Decapitation of fly heads was achieved by freezing 1.5ml Eppendorf tubes of whole flies in liquid nitrogen for 10min followed by 2min of vortexing and the heads were collected under the microscope. Homogenates were prepared by manual grinding of fly heads in sterilized 1.5ml Eppendorf tubes with a plastic pestle. Homogenates were prepared by processing 5 fly heads in 50µl of ice-cold TE buffer (20mM Tris-HCl, pH8, 1mM EDTA) containing protease inhibitor (1mM Phenylmethylsulphonylfluoride (PMSF, Melford Ltd, UK)), followed by 2 min sonication in the Ultrasonic bath. The homogenate was then centrifuged at 10,000g for 10 min. The supernatant was isolated and protein concentrations were determined with Bradford assay (See Section 2.4.3). Assays were carried out in triplicate.

2.6.4. Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

A 10% separating polyacrylamide gel, was prepared in a total volume of 10ml made up of 10µl of tetramethylethylenediamine (TEMED) and 100µl of 10% (w/v) ammonium persulphate (APS), added to a solution containing 30% (w/v) acrylamide, 10% (w/v)

SDS and 1.5M Tris (pH 8.8). A 5% stacking gel was also prepared in a total volume of 5ml made up of 5µl of TEMED and 50µl of 10% (w/v) APS, added to a solution containing 30% (w/v) acrylamide, 10% (w/v) SDS and 0.5M Tris (pH 6.8). The SDS-PAGE gel was then prepared in a Bio-Rad gel casting unit using 5ml of the 10% separating gel and 2ml of the 5% stacking gel. 5µg of protein extracts were diluted in a 3:1 ratio with 4x Laemmli sample buffer (BioRad Inc, US) containing 10% (v/v) β-mercaptoethanol (BME) placed in a 1.5ml Eppendorf tube and denatured by heating at 99^{o}C for 5 min. Denatured samples were loaded onto a 10% prepared SDS-PAGE gel with running buffer and run at 120V for 90 min in a Bio-Rad Mini Protean III gel system. 10µl of pre-stained molecular weight protein markers (BioRad Inc, US) were also loaded into a well as a guide to determine the molecular weight of the protein samples. Where indicated, gels were stained with InstantBlue® (Novexin Ltd, UK) for the detection of the protein bands.

2.6.5. Western blot

Once SDS-PAGE was completed, proteins were transferred onto a nitrocellulose membrane (GE Healthcare Ltd, UK) using the Trans-Blot® Turbo™ transfer system (BioRad, Inc, US) for 3min. Following transfer, Ponceau S solution (Sigma, US) was used to confirm effective transfer and protein loading. The membrane was then blocked with blocking buffer (5% non-fat milk for G proteins and 5% BSA for β-actin) for 90min at room temperature and incubated with the polyclonal primary antibody overnight at 4^{o}C at the following dilutions G_q and G_i, 1:500 and β-actin, 1:1000 in their respective blocking buffers. The membranes were then washed three times with TBST over a period of 15min. Polyclonal secondary antibodies were applied to the membrane at a dilution of 1:1000 for G_q,1:5000 for β-actin and G_i for 90min at room temperature. All antibodies were diluted in blocking buffer (5% non-fat milk or 5% BSA). The

membranes were then washed three times with TBST over a period of 15min. Chemiluminescent signals were developed using Pierce ECL western blotting substrate (Thermoscientific, UK) and captured by the ChemiDoc™ MP system. Image was analysed using Image Lab™ 4.1 software.

2.7. Data analysis

All data analysis was carried out using GraphPad Prism Software (version 4.0; GraphPad Software Inc., US). Statistical analyses were carried out using Student's T-test or Analysis of the Variance (ANOVA), followed by a suitable post-hoc test (Bonferroni post-test). For the gene expression studies, relative expression data were obtained using the comparative method ($2^{-\Delta\Delta Ct}$) (Schmittgen and Livak, 2008). To calculate relative gene expression, the data was normalised to the housekeeping gene, β-actin, $2^{-\Delta Ct}$ = (Ct β-actin – Ct G protein) for control and treated flies (Appendix 2.3). Ct is defined as the threshold cycle where fluorescent signal increases significantly. The numerical value of Ct is inversely related to the amount of amplicon in the reaction. Values of $P<0.05$ were considered to be significant.

Chapter 3: Behavioural Responses of *Drosophila* to Ethanol

Summary

This chapter describes experiments to characterise and quantify some of the behavioural responses of *Drosophila* to alcohol and it also quantifies the sedating effects of ethanol in flies. The approach taken is to use wild-type flies in a series of behavioural experiments to test ethanol sensitivity and ethanol tolerance induced by repeated exposure. Section 3.1. introduces the rationale for studying acute ethanol sensitivity, as well as tolerance. Section 3.2 describes the experimental assays used and provides the analysis of the results while Sections 3.3 and 3.4 are the discussion and conclusion related to this section of the study.

3.1. Introduction

3.1.1. *Drosophila melanogaster* as a model for studying ethanol-induced behaviour

Drosophila melanogaster encounter significant levels of ethanol in its natural environment (Kaun *et al*., 2012), being commonly found near rotting fruits and other plant materials that contain up to 5% ethanol, produced by fermentation of sugars by several yeasts (Guarnieri and Heberlein, 2003; Rodan and Rothenfluh, 2010). Female flies prefer to lay their eggs in food containing ethanol and flies can metabolise ethanol efficiently; they use it as an energy source and as a substrate for lipid biosynthesis (Devineni and Heberlein, 2013). As in mammals, the primary metabolising pathways in flies involve the conversion of ethanol to acetaldehyde by the enzymes alcohol dehydrogenase (ADH), and acetaldehyde dehydrogenase (ALDH). The functions of both ADH and ALDH are crucial to promoting resistance to ethanol toxicity (Guarnieri and Heberlein, 2003).

Drosophila is a powerful genetic model to investigate the molecular mechanisms underlying ethanol-induced behaviours (Guarnieri and Heberlein, 2003). This is justified by the fact that flies exhibit several behaviours similar to acute intoxication (loss of postural control or sedation) in mammals when exposed to ethanol vapour. The availability of powerful tools for genetic manipulation and the high degree of conservation at its genomic level make *Drosophila* a suitable model for ethanol-related behaviour studies (Heberlein, 2000).

Ethanol- induced behaviours in *Drosophila* have been investigated using mutated genes suspected to be involved in the ethanol response (Berger *et al*., 2008; Moore *et al*., 1998; Wen *et al*., 2005). For example, the *rutabaga (rut)* line displays altered ethanol sensitivity and carries a mutation in the gene encoding a calcium/calmodulin-dependent adenylyl cyclase (Moore *et al*., 1998); the mutant, *happyhour (hppy)* has reduced sensitivity to ethanol (Corl *et al*., 2009); while mutations in *neuropeptide F* (NPF, homologous to the mammalian neuropeptide Y) and its receptor NPFR-1 were shown to be involved in reduced ethanol sensitivity compared with wild-type flies (Wen *et al*., 2005). The *cheapdate* line carries a mutation in *amnesiac*, which encodes a neuropeptide, which is thought to activate the cAMP-signalling pathway and has been implicated in increased ethanol sensitivity (Moore *et al*., 1998).

3.1.2. Measuring ethanol induced behaviours

Different methods have been developed in the past years to determine the effects of ethanol on *Drosophila's* behaviour (Table 1.2). Such behaviours include ethanol sensitivity (Berger *et al*., 2008; Bhandari *et al*., 2009; Devineni *et al*., 2011; Dzitoyeva *et al*., 2003, Kong *et al*., 2010; Maples and Rothenfluh, 2011; Moore *et al*., 1998; Rothenfluh *et al*., 2006); ethanol tolerance following initial ethanol exposure (Berger *et*

al., 2004; Berger *et al*., 2008; Chan *et al*., 2014; Cowmeadow *et al*., 2006; Krishnan *et al*., 2012; Maples and Rothenfluh, 2011; Scholz *et al*., 2000); ethanol preference (Devineni and Heberlein, 2009) and conditioned preference (Kaun *et al*., 2011; Shohat-Ophir *et al*., 2012) behaviours that model specific features of addiction. Studying such ethanol responses may provide an understanding into the mechanisms regulating more complex addiction-related behaviours.

3.1.2.1. Acute ethanol sensitivity

Acute ethanol response refers to the initial response to the first exposure to ethanol *Drosophila* has experienced. During acute exposure to ethanol vapour, flies exhibit behaviours similar to those seen in rodents and humans; low doses of ethanol induces a state of increasing activity, whereas higher doses lead to decreased activity, eventual loss of postural control and then sedation (Moore et al., 1998; Singh and Heberlein, 2000). Ethanol vapour has been used as an effective method to deliver a reproducible ethanol dose and to rapidly sedate flies (Cowmeadow et al., 2005; Maples and Rothenfluh, 2011; Moore et al., 1998; Wen et al., 2005). When flies are exposed to ethanol vapour, they first become hyperactive and display response behaviours such as walking fast and vibrating their wings. Different types of assays have been developed to measure ethanol sensitivity in flies.

3.1.2.2. Ethanol Tolerance

Alcoholics develop tolerance to the intoxicating effects of ethanol after initial consumption, which is usually associated with dependence and uncontrolled craving to continue drinking (Diamond and Gordon, 1997). Different types of ethanol tolerance have been characterized in *Drosophila* (Chapter 1). Two mechanisms of tolerance have

been reported in *Drosophila*: metabolic/pharmacokinetic tolerance, which involves 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 Atkinson, 2009).

Previous studies have shown that *Drosophila* can acquire rapid tolerance once exposed to the sedating effects of ethanol (Scholz *et al*., 2000). Thus in flies, many genes have been implicated in ethanol tolerance. For instance, flies carrying the *slowpoke gene* , a Ca^{2+} activated K^{+} channel gene which is critical modulator of neuronal excitability, have been shown to be required for the acquisition of ethanol tolerance (Cowmeadow *et al*., 2005; Cowmeadow *et al*., 2006). Flies that carry the *hangover (hang)* gene (a gene encoding a nucleic acid binding zinc finger protein) were implicated in reduced tolerance development in the same manner as flies lacking the neuromodulator, octopamine owing to a mutation in the gene encoding tyramine B hydroxylase (Scholz *et al*., 2000; Scholz, *et al*., 2005). The *Drosophila* homologue of the *jwa gene* (encoding a large Prenylated Rab Acceptor 1 (PRA1)) (Li *et al*., 2008) and the *shibire gene* (encoding for the *Drosophila* dynamin) (Krishnan *et al*., 2012), were also reported to be necessary for the development of ethanol tolerance. Finally, long term memory mutants including *exba (elF-5C)* (translational regulator), *formin3* (invloved in actin assembly) and *pumilio* (translational regulator) have been implicated in reduced ethanol tolerance (Berger *et al*., 2008).

3.1.2.3. Ethanol preference and conditioned preference

Assays have been developed to measure ethanol self-administration in *Drosophila* using the capillary feeder assay (CAFE). This assay demonstrates that flies prefer to consume ethanol-containing food to regular food, indicating compulsive consumption of ethanol (Devineni and Heberlein, 2009; Shohat-Ophir *et al*., 2012). To determine whether ethanol administration is rewarding to flies, a conditioned preference assay was developed by Kaun *et al*., (2011). In this assay, flies are exposed to two neutral odour cues at first, one of which is paired with a moderate exposure to ethanol vapour. Flies are later offered a choice between the two odours and preference for ethanol-associated odour is measured.

3.1.3. Objective

The aim of this chapter is to quantify selected ethanol-induced behavioural responses in age-matched (1 - 3 days old) *Drosophila* such as ethanol sensitivity and tolerance and also to identify subpopulations (early and late responders) of *Drosophila*, which were selected due to their responses to ethanol.

3.2. Results

3.2.1. Ethanol sensitivity assay

Ethanol sensitivity was carried out based on previous work (Maples and Rothenfluh, 2011). In the ethanol sensitivity assay, flies were placed in a 25 x 95mm food vial and trapped in the vial with a cotton-like plug (Figure 2.1). The ethanol solution (500µl) was added to the bottom of the plug (exposed side), with the ethanol facing into the vial. At one-minute intervals thereafter, flies were gently tapped to the bottom of the vial and then observed for 10s, for their ability to remain stationary in the continuous presence of ethanol vapour from the plug. Ethanol sensitivity was quantified as the time required for 50% of exposed flies to sedate (ST50) (Maples and Rothenfluh, 2011). This is a metric routinely extracted from similar ethanol sedation time-course studies (Chan *et al*., 2014). The number of flies that became stationary once exposed to ethanol vapour was recorded (see Section 2.2.1 for assay details). Note that lower and higher ST50s indicate increased and decreased ethanol sensitivity respectively (Chan *et al*., 2014).

To determine whether flies were sensitive to the sedating effects of ethanol and the appropriate dose to use in the ethanol sensitivity assay, an ethanol dose-response test was performed using age-matched (1 – 3 days old) eight male and female wild-type flies. The flies were collected the day before testing in a plastic food vial containing fresh food without yeast (the yeast tends to inhibit the growth of unwanted fungi but can produce ethanol). Ethanol solutions of 10, 30, 50, 70% were prepared by mixing ethanol and water at a ratio (vol: vol) of 1:9, 3:7, 5:5 and 7:3 respectively and were used to measure the flies resistance to ethanol. When exposed to the different ethanol solutions, including 100% ethanol, flies became hyperactive, walking up and down the plastic vial, wings vibrating and gradually losing postural control. The ST50 is the time after the start of the experiment when four of the eight flies remain stationary. Both males and females became sedated in the presence of ethanol vapour (Figure 3.1). Female flies

exposed to 100% ethanol showed ST50 of 10.33±0.88min whereas it took longer with 70% (16.33±0.67min), and 50% (20.33±0.56min) (Figure 3.1).

The data for 10% and 30% ethanol were not included as it took > 1h for 10% ethanol and >31min for 30% ethanol to reach 50% sedation. As expected, sedation in the sensitivity assay was dose dependent (Chan *et al*., 2014), such that lower ethanol concentrations (50%) resulted in longer ST50. In order to choose a suitable exposure concentration, it was necessary to compare ST50s of all ethanol concentrations (excluding 10% and 30%) (Figure 3.1). 100% ethanol was chosen because it had the shortest ST50 with no recorded mortality.

In order to determine which sex of flies to use in all experiments, the ST50s in males and females were taken into account. Although, the differences were not statistically significant, males sedated more slowly than females, showing higher ST50. This difference appeared even stronger at a lower concentration of 50% ethanol, at which males showed an ST50 of 23.33 ± 0.88min, whereas the ST50 of females was 20.33 ± 0.56min (Figure 3.1). Males are generally more resistant to the sedating effects of ethanol than females (Devineni and Heberlein, 2012). Other investigators demonstrated that there was no significant difference between males and females (Maples and Rothenfluh, 2011). In all experiments, female flies were used to avoid sexual dimorphism, because female flies are larger in size compared to males.

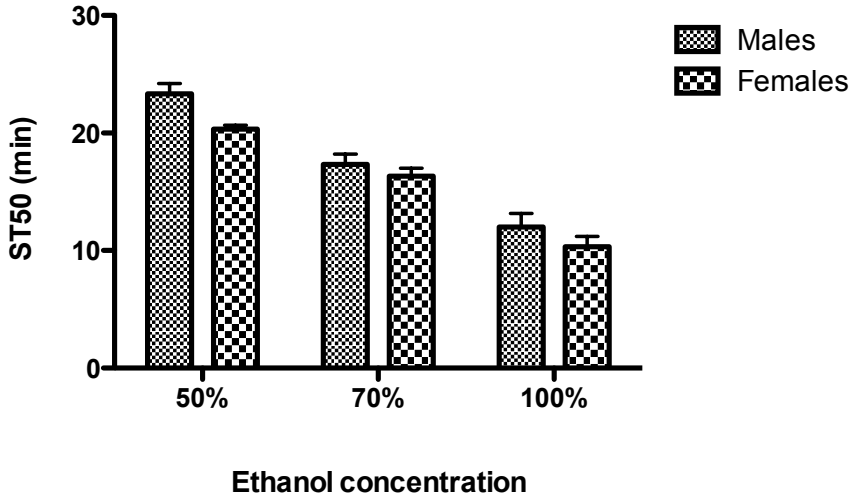

Figure 3.1. Dose response sedation of wild-type flies exposed to increasing ethanol concentration

Data are from male and female flies exposed to vapour from the indicated ethanol concentration (50, 70 and 100%). ST50 Two-way Analysis of Variance (ANOVA) analysis revealed a significant decrease in ST50 with increasing ethanol concentration (***P<0.001; n = 8 females; n = 8 males). Males showed a higher ST50 compared to females. Error bars represent ± Standard Error of the Mean (SEM). Data are representative of three independent experiments.

3.2.2. Ethanol-induced tolerance in wild-type *Drosophila*

Tolerance is defined as a reduction in ethanol sensitivity during a second exposure to ethanol following recovery from a prior exposure (Devineni *et al*., 2011; Scholz, 2009). To determine whether flies develop tolerance rapidly to the sedative effects of ethanol, female flies became sedated during the first, second exposure and third ethanol exposure separated by 24 h recovery in ethanol sensitivity assays described above using 500µl of 100% ethanol (see section 2.2.2 for assay details). There was a significant increase in ST50 between the first exposure and the third exposure, thereby indicating the flies have become less sensitive to ethanol. The ST50 during the first exposure was 11 ± 0.58min, whereas the ST50 of the third exposure was ST50 of 13.7 ± 0.88min (Figure 3.2A). The 24h recovery time point was used not only to ensure that flies had fully recovered from the sedating effects of ethanol but also to ensure they had

completely metabolised all the ethanol absorbed and had time to rehydrate and feed (Scholz *et al*., 2000).

To determine the number of ethanol exposures that will be required in all experiments, the number of ethanol exposures was extended by another exposure in another set of female flies. After four consecutive ethanol exposures with 24h recovery period, a significant increase was observed in ST50 between the first and third and the first and fourth exposure, but in the fourth exposure ST50 was lower than in the third exposure (Figure 3.2B). Thus, flies become more sensitive to ethanol, as they are exposed more than three occasions. For this reason, exposing flies three times will be used throughout the thesis.

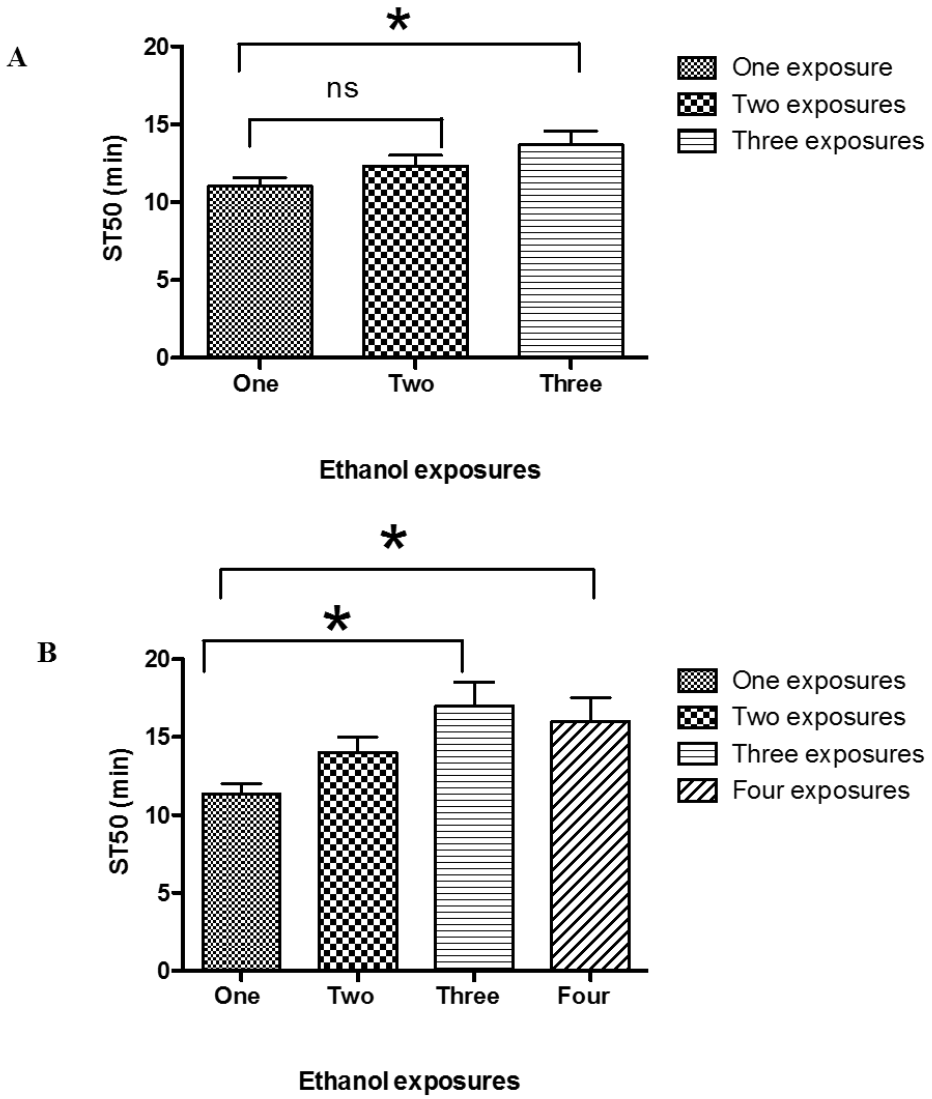

Figure 3.2. Ethanol tolerance in wild-type *Drosophila* in ethanol sensitivity assay

(A) Time to 50% sedation (ST50) from flies exposed once to 100% ethanol for three consecutive days and allowed to recover for 24 h, revealed a significant increase in one and three exposures (One-Way Analysis of Variance (ANOVA); *P<0.05; n = 8 female flies; ns = not significant). **(B)** ST50 values for flies exposed to ethanol for four consecutive days spaced by 24 h recovery period, increased significantly between one and three exposures and one and four exposures (One-Way Analysis of Variance (ANOVA) Bonferroni posttest; *P<0.05; n = 8 female flies). Error bars represent ± Standard Error of the Mean (SEM). Data are representative of three independent experiments.

3.2.3. Subpopulations of wild-type *Drosophila* (early and late responders)

The intial experiments suggested that there were individual variations in ethanol susceptibility among flies. Given that the aim of this project was to detect changes in gene expression levels, isolating fly populations with homogenous behavioural characteristics was a requisite. The aim of this experiment is to select two different populations of flies, breed and observe their behaviour with respect to ethanol consumption. Flies were divided into two groups to identify subpopulations (early and late responders) which may be susceptible to alcohol and addiction and to select a more homogenous population of wild-type flies. To the author's best knowledge, this is the first study demonstrating the generation of early and late responders to ethanol.

Age-matched (1 -3 days old) twenty male and virgin female flies were collected days before ethanol exposure. Virgin female flies were collected within 8 h once the culture vials are cleared of all adult flies (Section 2.1.3). On the day of the experiment, male or female flies were separately exposed to 500µl of 100% ethanol in the assay described in Chapter 2, Section 2.3.1. The first 20% of flies to be sedated were identified as the early responders whilst the last 20% of flies were identified as the late responders (Figure 2.2 of Chapter 2). The 20% cut-off point was used as an arbitary value to select flies displaying ethanol-induced characteristics. As expected, the early responders showed a lower sedation time compared with the late responders for both male and virgin female flies (Figure 3.3A & B). These sets of flies were identified as the parental generation. In order to explore the basis of different ethanol responses, a classical experimental cross approach was carried out. The parental generation of early male (EM) and early female (EF) responders and the parental generation of late male (LM) and late female (LF) responders were bred separately in a plastic food vial containing yeast (See section 2.3.2 for assay details). 10 days later, the offsprings of the parental generation (known as the

F1 generation), males and virgin females were collected and the process is repeated as described above to create the F2 generation (offspring of the F1 generation).

The sedation time values of both male and female flies were combined in the analysis of early and late responders. In the F1 generation (offspring of the parental generation), there was a significant difference observed between all early (F1-EM x EF) and late (F1-LM x LF) responders to ethanol. The ST50 of the F1 generation in the early responders were shorter than the late responders (Figure 3.4). Whereas, in the F2 generation (offsprings of the F1 generation), a significant difference was also observed between all early (F2-EM x EF) and late responders (F2-LM x LF). The early responders also displayed a lower ST50 compared to the late responders (Figure 3.4).

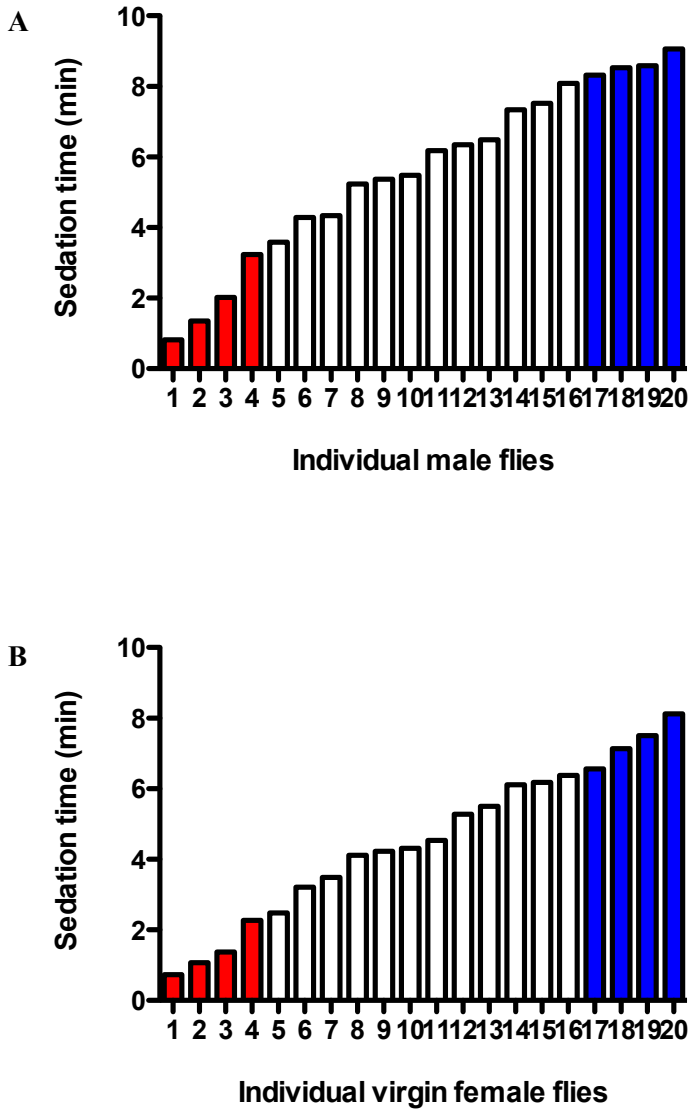

Figure 3.3. Ethanol sedation time of male and virgin female flies exposed to 500µl of 100% ethanol

Values were derived in response to ethanol exposure in both males (A) and virgin females (B). The first 20% (in red) were selected as the early responders. The last 20% of flies (in blue) were selected as the late responders. Data are representative of one experiment.

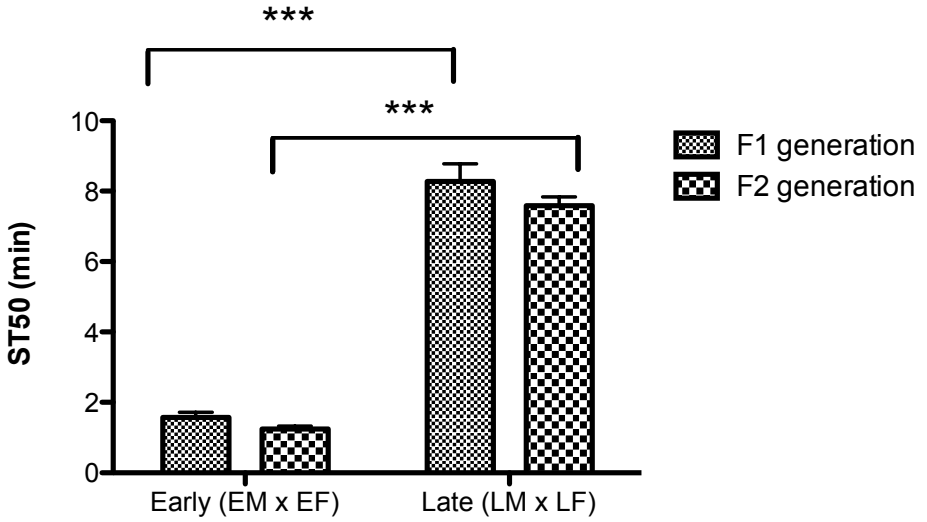

Figure 3.4. The F1 and F2 generation of early and late responders to ethanol

Ethanol sedation time (ST50) of early and late responders (all male and female flies) for both the F1 and F2 generation. ST50 in early responders were shorter than late responders (n= 8 males, n= 8 females). Error bars represent ± SEM and asterisks denote statistical significance by Two-way ANOVA, Bonferroni posttest, ***P<0.001. Data are representative of three independent experiments.

3.2.4. Ethanol-induced tolerance in subpopulations of wild-type *Drosophila* (early and late responders)

Ethanol tolerance was assessed in the parental generation of early and late responders to address the potential of genetically influenced flies on repeated ethanol exposures. Early and late responders (female flies) from the parental generation, collected the day before the ethanol exposures, were exposed daily to 500µl of 100% ethanol for three consecutive days in the ethanol sensitivity assay, spaced by 24h recovery period as described previously. During the first ethanol exposure, late responders sedated more slowly than early responders showing a higher ST50: 10.33 ± 0.88min, whereas early responders showed ST50 of 7.27 ± 0.37min (Figure 3.5). Thus as expected from Figure 3.4, late responders are less sensitive to the sedative effects to ethanol than the early responders as they sedate more slowly and recover more quickly.

The difference between the early and late responders appeared be maintained throughout the three ethanol exposures. ST50 increased from one to three ethanol exposures, thereby indicating that both subpopulations of wild-type flies (early and late responders) developed resistance to the sedating effects of ethanol. For this reason, total alcohol dehydrogenase was next measured in early and late responders.

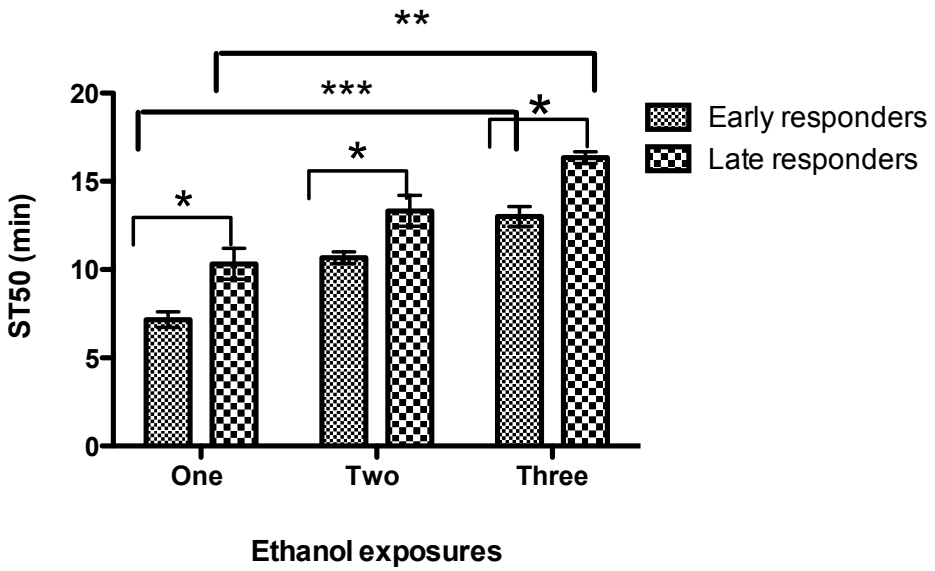

Figure 3.5. Ethanol tolerance in subpopulations of wild *Drosophila* (early and late responders)

Time to ST50 (min) of early and late responders during repeated ethanol exposures. The difference between early and late responders was maintained during the three exposures. There was a significant increase in ST50 in both groups of flies during the three days of exposure ($P<0.05$) (Two-way Analysis of Variance Bonferroni posttest; * $P<0.05$, ** $P<0.01$, *** $P<0.001$; n = 8 female flies). Error bars represent ± SEM. Data are representative of three independent experiments.

3.2.5. Ethanol metabolism in subpopulations of wild-type *Drosophila* (early and late responders)

To determine whether the effects produced by early and late responders above could be as a result of a difference in ADH activity, the activity of ADH was measured. As in

mammals, the primary metabolising pathways in flies involves the conversion of ethanol to acetaldehyde by the enzyme alcohol dehydrogenase (ADH) and further conversion to acetate by the mitochondrial acetaldehyde dehydrogenase (ALDH) (Sha *et al*., 2014, Zakhari, 2006). ADH is of particular importance in *Drosophila*, because it feeds on fermenting plant material (Geer et al., 1989).

Alcohol dehydrogenase activity was measured based on previous work (Pfeiler *et al*., 2005). To test this, using wild-type flies as control, parental generation of naïve early and late responders (not exposed to ethanol) were collected the day before and assayed for ADH activity. There was no significant difference observed between early and late responders (Figure 3.6).

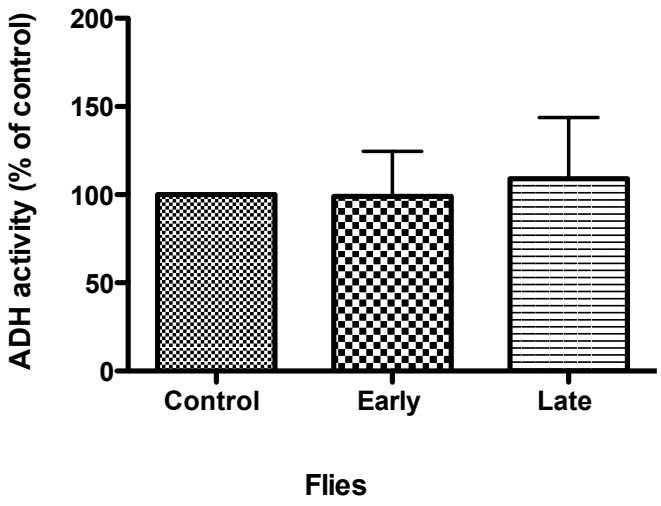

Figure 3.6. ADH activity in early and late responders

ADH specific activity in female early and late responders. Late responders showed a higher alcohol dehydrogenase activity compared to control (wild-type) and early responders. This increase in ADH activity was not significant (Unpaired Student's T-test, $P>0.05$, $n = 8$ female flies each). Error bars represent ± Standard Error of the Mean (SEM). Data are representative of three independent experiments.

3.3. Discussion

The fruit fly, *Drosophila melanogaster* has been used as a suitable model for studying acute and chronic responses to ethanol (Devineni *et al*., 2011). Flies show a complex and biphasic behavioural response upon exposure to ethanol vapour; an initial increase in locomotion, loss of postural control, and eventually sedation (Devineni and Heberlein, 2010). Previous studies have examined and characterised ethanol sensitivity, preference and tolerance using several assays. *Drosophila* has been shown to have preference for ethanol-containing over non-ethanol containing food and that this preference is known to increase with increasing ethanol concentrations (Devineni and Heberlein, 2009). Secondly, flies rapidly return to high levels of ethanol consumption after a period of imposed abstinence (Devineni and Heberlein, 2009). Thirdly, flies develop tolerance to the sedating effects of ethanol similar to those in mammals (Scholz *et al*., 2000).

In this study, ethanol sensitivity assay measuring the fly's resistance to the sedating effects of ethanol was employed (Maples and Rothenfluh, 2011). The assay involved the use of different methodologies in quantifying ethanol sensitivity, which included measuring the time it takes for half of exposed flies to become stationary. When exposed to the sedating effects of ethanol, flies displayed behaviour similar to that of mammals, including humans. As observed by Wolf and Heberlein (2003), flies absorbing ethanol vapour showed a brief increase in walking velocity; they lost their ability to stand or walk properly and eventually lost postural control. In summary, ethanol exposure causes clear and measurable effects on *Drosophila* locomotion and postural control. Additionally, the concentrations of ethanol that causes increased locomotion in *Drosophila* are very similar to that observed in mammals (Wolf and Heberlein, 2003).

Using the ethanol sensitivity assay, sex differences were measured in *Drosophila*. Although, not statistically significant, male flies were less sensitive to ethanol sedation than females. Women have been reported to be more sensitive than men to ethanol-induced sedation and this difference is partly due to differences in blood alcohol content (Miller *et al*., 2009) or ethanol pharmacokinetics and partly to sex differences within the nervous system (Devineni and Heberlein, 2012). Furthermore, women are more susceptible to the negative physical consequences of heavy drinking such as organ damage and risk of death (Nolen-Hoeksema and Hilt, 2006). Previous studies did not observe sex differences in *Drosophila* ethanol sensitivity (Bhandari *et al*., 2009; Maples and Rothenfluh, 2011). This discrepancy may be as a result of the behavioural assay used, in the case of Bhandari *et al*., a negative geotaxis assay was used, which involves the loss of negative geotaxis that is not a direct correlation of ethanol sedation. Whereas in Maples and Rothenfluh, because of the modification of the exposure chamber, which does not have fly food before inserting the plug in the vial, differences were observed in results obtained.

The individual differences in ethanol sensitivity in flies led to the investigation of how flies respond to ethanol exposure. Subpopulations (early and late responders) of wild-type *Drosophila* were selected and separated based on their response to the sedating effects of ethanol. Early responders are more sensitive to ethanol exposure than late responders and this difference may be attributable to a difference in ethanol pharmacokinetics. A previous study has observed differences in alcohol response in rats (Bell *et al*., 2006), thereby associated with addiction.

In addition to the isolation of early and late responders, the difference in ethanol response was investigated by carrying out genetic crosses between the F1 and F2 generations. The F2 generation displayed similar behaviour to the F1 generation. However in both cases, the F1 generation were less sensitive than F2 generation as

observed by their loss of postural control and sedation. These findings suggest that there is a genetically based difference in alcohol sensitivity among wild-type flies and when isolated, the offspring from the population seem to maintain the same alcohol sensitivity as the parents.

It is well known that *Drosophila* develop tolerance to the sedating effects of ethanol following a prior exposure. Previous studies have examined and characterised ethanol tolerance in *Drosophila* using various assays (Berger *et al*., 2004; Chan *et al*., 2014; Cowmeadow *et al*., 2005; Cowmeadow *et al*., 2006; Devineni *et al*., 2011; Dzitoyeva *et al*., 2003; Maples and Rothenfluh, 2011; Scholz *et al*., 2000). For instance, Scholz *et al* (2000) and Berger *et al* (2004) characterised rapid and chronic tolerance in the inebriometer and recovery assay. However, none of these studies have characterised tolerance for the assay used in this investigation. In this study and using the ethanol sensitivity assay, the quantitative aspects of ethanol tolerance in flies, such as the observed behaviour are similar to those previously described (Maples and Rothenfluh, 2011). Taken together, these data reinforce that the sensitivity assay is a suitable model in which to measure ethanol tolerance in *Drosophila*.

It is however, important to contrast the tolerance assay in this study with that of previous studies. The tolerance assay differs in that it measures chronic ethanol sensitivity in response to three/four consecutive treatments, allowing 24 h recovery period. The assay has been extended to measure the flies acquired resistance or tolerance to the effects of ethanol on loss of postural control and locomotion rather than sedation. It was also extended to measure the limit to which the flies can become tolerant rapidly to ethanol by measuring the acute response of these flies (one ethanol treatment) and chronic response (two and three ethanol treatments).

Using the ethanol sensitivity assay, wild-type flies exposed to ethanol repeatedly for three consecutive days, displayed increased ethanol tolerance. This finding correlates

with flies exposed on four consecutive days, also displayed increased resistance to ethanol. Consumption of ethanol produces lasting physiological changes, including tolerance and in some individuals, addiction (Cowmeadow *et al*., 2005). Ethanol tolerance is defined as acquired resistance to the behavioural effects of ethanol due to repeated ethanol exposure (Devineni and Heberlein, 2013).

Tolerance, induced by repeated ethanol exposure was also measured in early and late responders. The findings indicated that there was a difference between early and late responders during one and three ethanol treatments. The difference between both groups of flies was maintained throughout the ethanol exposures. These differences observed may also be due to epigenetics differences and studying these individual differences in humans for example, could help provide an understanding into individualised treatment of addiction (Baker *et al*., 2014). There was relatively little variation between repeated measures of ST50 but there are differences observed between the selected populations of flies (early and late responders). This highlights the advantage of ST50 that eliminates the outliers for a mixed population.

Drosophila melanogaster have been shown to contain alcohol dehydrogenase (ADH) (Geer *et al*., 1988), an enzyme that metabolises ethanol. This study also showed that there was no significant difference in the level of ADH between early and late responders. This non-significant result suggests that the ADH may not be the only reason for this observation and that other factors may be associated such as aldehyde dehydrogenase (ALDH), which is involved in the metabolism of acetaldehyde to acetate. Previously, investigators have studied ADH activity in male and female flies and have reported decreased ADH activity in females compared to males (Pipkin and Hewitt, 1972, Geer *et al*., 1988) but not all studies (Barbancho *et al*., 1987) have observed this difference. In conclusion, the lack of significant differences in ADH

between early and late responders does not support the hypothesis that late responders could be metabolising ethanol faster than early responders.

3.4. Conclusion

In this chapter, ethanol sensitivity and tolerance have been validated in *Drosophila melanogaster* as a relevant model for human ethanol studies. The findings here confirm previous reports that flies develop sensitivity to the sedating effects of ethanol (Corl *et al*., 2009; Devineni *et al*., 2011) and when exposed repetitively to ethanol, they develop ethanol tolerance (Chan *et al*., 2014; Cowmeadow *et al*., 2006; Scholz *et al*., 2000). Individual flies displayed variations in their sedation times and this may be due to their susceptibility to ethanol. This led to the isolation of subpopulations of *Drosophila*, early and late responders. This therefore suggests that among the wild-type population, there is a genetically based variation in the response to ethanol. The next chapter will demonstrate changes in G protein gene expression in wild-type *Drosophila* following repeated exposures to ethanol.

Chapter 4: G protein gene expression changes in wild-type *Drosophila*

Summary

This chapter describes experiments designed to characterize the effect of ethanol-induced behaviour on G proteins. The approach taken is to use wild-type *Drosophila* as a model to investigate whether changes on G protein gene expression as measured using real-time polymerase chain reaction can be correlated to tolerance. Section 4.1. introduces the rationale for studying changes in gene expression and alcohol addiction. Section 4.2 describes the experimental assays used and provides the analysis of the results while section 4.3 and 4.4 is the discussion and conclusion of this part of the investigation.

4.1. Introduction

Drosophila has been shown to develop sensitivity and tolerance to the sedating effects of ethanol (Chan *et al*., 2014, Devineni *et al*., 2011, Scholz *et al*., 2005) and was confirmed with the experiments described in Chapter 3. Ethanol produces both positive and negative effects in humans. Consuming and abusing ethanol have enormous health and socioeconomic impact on the world population (Spanagel, 2009). Ethanol affects a number of receptors such as NMDA and $GABA_A$, and voltage dependent channels (reviewed by Erdozain and Callado, 2014). Acute use of ethanol has been reported to cause cellular changes in the brain lasting for hours whereas chronic use causes extensive neuroadaptive changes in the central nervous system that are long-term. This involves the remodelling of synapses that are dependent on changes in gene expression during chronic alcohol use (Most *et al*., 2014). The identification of these gene

expression changes are of critical importance for the understanding of addictive behaviour.

Both short and long-term effects of ethanol can be associated with the modulation of the seven transmembrane G protein-coupled receptors (GPCR) (Most *et al*., 2014). G proteins play a crucial role in linking GPCR binding to changes in specific intracellular signalling pathways and in describing the specificity and temporal characteristics of a cellular response (Oldham and Hamm, 2008). G proteins are versatile signalling molecules. They consist of three subunits: α, β and γ. The α subunit is the most important component for the activation of specific signalling cascades (Neer 1997). Due to the effect of ethanol on other neurotransmitter systems, the expression of G proteins may also be affected by the drug. The effect of chronic ethanol on the expression of G protein subunits has been studied at protein level (Chapter 1, Section 1.33). Despite the fact that initiation of a signalling cascade seems to be of vital importance to the induction of long-term changes by abused drugs, the literature on the effects of chronic ethanol administration on G proteins is rather limited.

It is therefore important to establish whether G protein mRNA expression levels are altered by ethanol administration because changes in G protein expression could affect cellular signalling pathways and thereby contribute to the molecular mechanisms underlying some of these adaptive responses to ethanol exposure. It is also important to measure G protein expression changes using western blot. How G proteins play a role in alcohol addiction via these pathways is yet to be understood. These findings will hopefully open new avenues for potential therapy of alcoholic neurological disorders and the identification of candidate genes that may be altered in genetic alcoholism.

4.1.1. Objective

In previous studies, G protein gene expression changes have been shown using different drugs of abuse such as psychostimulants (amphetamines and cocaine), opiates (morphine) and barbiturates. Although, the effect of ethanol on the cAMP-PKA signalling pathway has been demonstrated, no studies have been carried out on ethanol-induced changes on G protein gene expression. In Chapter 3, it was reported that wild-type and subpopulations (early and late responders) *Drosophila* develop tolerance to the sedative effects of ethanol. This chapter will present data from experiments using wild-type and subpopulation (early and late responders) *Drosophila* to investigate gene expression changes in *Drosophila* G protein subunits (Gα, Gβ and Gγ) following repeated exposures to ethanol. The aim is to investigate whether ethanol-induced behaviour might alter G proteins expression.

4.2. Results

4.2.1. G protein genes are expressed in the head of wild-type *Drosophila*

In order to verify if G protein subunits are expressed in the head of *Drosophila*, it was necessary to design and validate the use of a set of primers for each of the known *Drosophila* genes that encode for G protein subunits, Gα (G_i, G_s, G_o, G_q, G_f and cta), Gβ ($G\beta_{13F}$, $G\beta_{76C}$ and $G\beta_5$) and Gγ ($G\gamma_{30A}$ and $G\gamma_1$). Each G protein subunit had more than one transcript except the $G\beta_5$ subunit. To design primers, the transcripts for each gene were aligned first using a free online tool known as CLUSTALW2. Using primer-BLAST on National Centre for Biotechnology Information (NCBI), the primer pairs were designed and selected to span an exon-exon junction to distinguish potential genomic DNA amplification (See Section 2.5.2 for details).

To investigate if each of the G protein genes were expressed in *Drosophila*'s head, RNA was extracted from the head of naïve flies and was tested using the one-step reverse transcriptase polymerase chain reaction (RT-PCR). Negative controls were included in the assay to detect potential contamination [no template control (NTC) i.e. no RNA] and possible genomic DNA contamination [minus reverse transcriptase control (-RT)]. The results of the RT-PCR amplification for the six genes that encode for the Gα subunits (G_i, G_q, G_f, G_o, G_s and cta), are shown in Figure 4.1A. All the Gα subunits were expressed in the fly's head and the RT-PCR products were all found to be of the predicted molecular size. No bands were observed in both negative controls, thereby indicating the absence of genomic DNA contaminations, primer-dimer formation or other sample contaminations.

In the *Drosophila* genome, three genes encode for Gβ subunits ($G\beta_{13F}$, $G\beta_{76C}$ and $G\beta_5$), the results of the RT-PCR amplification are displayed in Figure 4.1B. $G\beta_{13F}$ and $G\beta_{76C}$ were expressed in the head of *Drosophila* and no bands were observed in both negative controls. Due to the lack of introns in the $G\beta_5$ gene, it was not possible to design a primer pair to span the exon-exon junction. However, given the lack of signal from potentially contaminating DNA in the amplification products of the other primer sets, the identified RT-PCR products for $G\beta_5$ can be considered an amplification of mRNA.

Figure 4.1C shows the RT-PCR results for the two genes that encode Gγ genes, $G\gamma_{30A}$ and $G\gamma_1$. These findings are consistent with previous study, which demonstrated that G protein subunits and their variants are expressed in *Drosophila* head and olfactory receptor organs (Boto *et al.*, 2010).

To fully confirm if the G protein subunits were free of possible genomic contamination, Figure 4.2 depicts a full agarose stained gel of one Gα subunits (G_s). The image shows that the gel is free of DNA contamination and with an intron size of 1415bp between the exons of G_s gene and under the PCR condition used; there wouldn't be another band

other than the gene of interest because the size of the intron is too large to be amplified. This is shown as an example and the same was true for all other amplification products (Appendix 4.1).

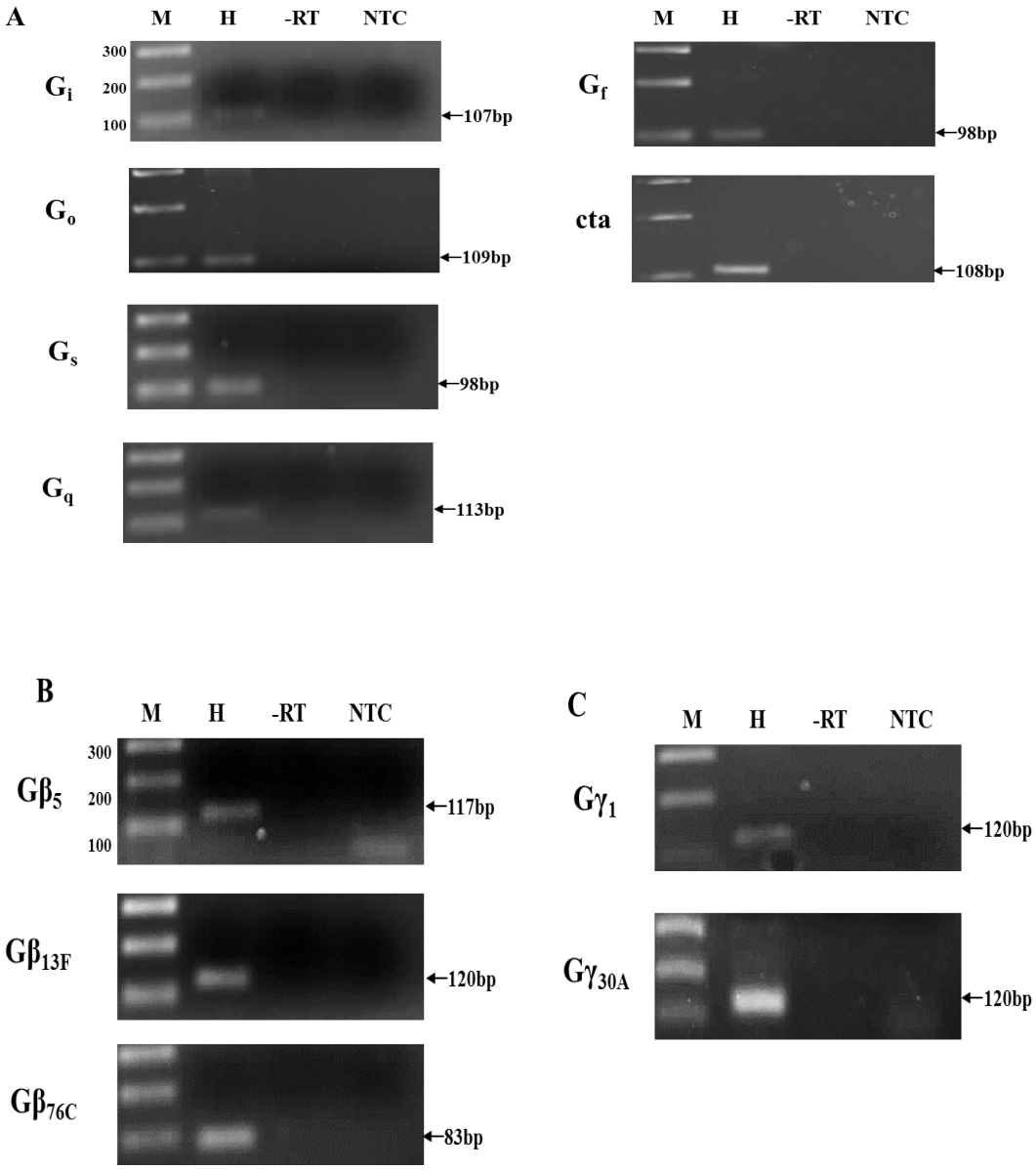

Figure 4.1. Expression analysis of G protein subunits in the head of adult *Drosophila* using reverse transcriptase chain reaction (RT-PCR)

Target genes are denoted on the left of the figure and the amplification product size on the right, expressed in base pairs (bp). RNA was extracted from the head of the flies and this is identified at the top of each figure as follows: M: 100bp ladder; H: head; -RT (minus reverse transcriptase); NTC (No template control). (A) Gα expression (B) Expression results for Gβ subunits (C) Expression of Gγ subunits.

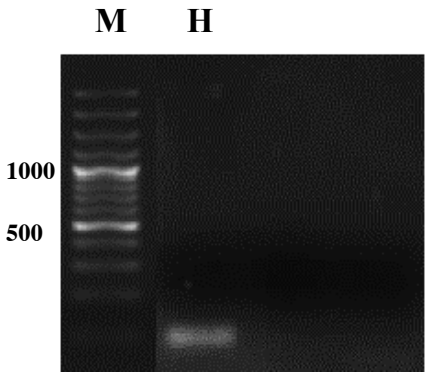

Figure 4.2. Agarose gel demonstrating that no other RT-PCR products were found except in the predicted molecular region

M- 100bp ladder on the right, H – Head

4.2.2. Effect of ethanol on G protein gene expression

Ethanol is a highly addictive psychomotor stimulant that elicits a state of enhanced arousal, reward and disinhibition (Nestler, 2004). One of the main objectives of this study is to identify changes in G protein expression that correlate with the observed ethanol-induced behavioural changes. In Chapter 3, it was confirmed that the flies respond to ethanol (sensitive to the effects of ethanol) and that on consecutive exposures, the time to 50% sedation increases (tolerance). Accordingly, G protein gene expression was measured at the following time points: i) in flies never exposed to ethanol (referred as: naïve flies), ii) after the first exposure to ethanol (referred as: acute response in naïve flies), iii) in flies exposed twice to ethanol where the last exposure was 24h prior to sacrifice (referred to as basal level in chronic flies), iv) after the third exposure (referred to as acute response in chronic flies). The flies were sacrificed 1h after the last ethanol exposure. This time point (1h after ethanol exposure) was chosen to measure the acute response of ethanol following tolerance and also to allow gene expression changes to occur. This protocol described in Table 4.1 was maintained for the whole study.

G protein mRNA levels were measured in the pooled heads of wild-type flies treated in the time points as described above. mRNA levels were measured using quantitative real-time polymerase chain reaction (qRT-PCR) (Chapter 2 for details). Non-treated flies were used as a control for gene expression.

An initial screen of all Gα subunits was carried out and the two that showed the most consistent changes were the G_i and G_q subunits (Appendix 4.2) and were further investigated. Ethanol exposure induced reduced G_q mRNA expression in all ethanol exposure time points. A slight increase was observed in both the acute response in naïve and chronic flies and a decrease in the basal level in chronic flies in the mRNA

expression of G_i subunit. An increase in G_s mRNA expression was observed in all ethanol exposures (Figure 4.3).

Table 4.1. Ethanol-induced treatment model

Exposure group	Day 1	Day 2	Day 3
No ethanol (Naïve control flies)	----	----	----
One (Acute response in naïve flies)	----	----	Ethanol and sacrificed 1h later
Two (Basal level in chronic flies)	Ethanol	Ethanol	Sacrificed
Three (Acute response in chronic flies)	Ethanol	Ethanol	Ethanol and sacrificed 1h later

All flies were exposed for 30 min to rapidly induce tolerance allowing 24h recovery period and sacrificed on Day 3 1 h after the ethanol exposure.

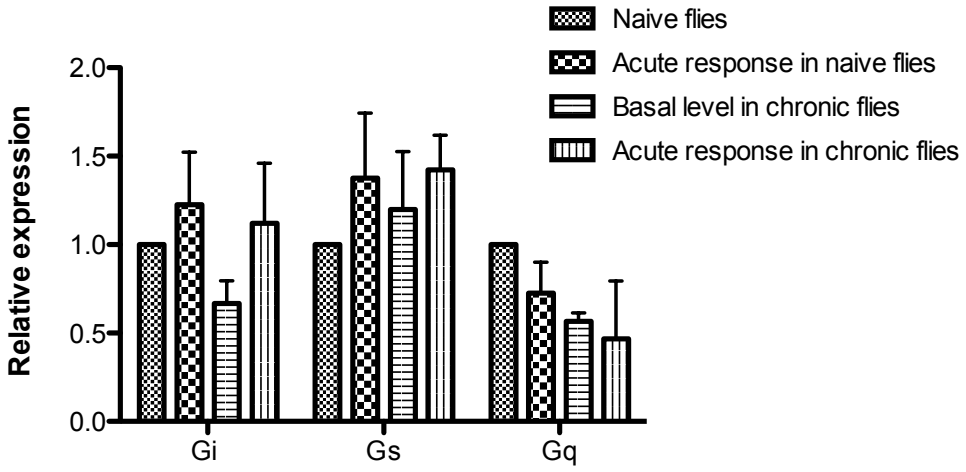

Figure 4.3. Relative Gα genes, Gi, Gs and Gq genes in the head of wild-type flies

Flies were exposed as follows: one (acute response in naïve flies), two (basal level in chronic flies) and three (acute response in chronic flies) and sacrificed 1 h after the last ethanol exposure except for flies exposed twice, which were killed 24 h after the last exposure. Relative mRNA expression (relative to naïve flies –control). Error bars represent ± Standard Error of the Mean (SEM) (Student's T-test, $P<0.05$; n=5 female flies). Data are representative of two independent experiments.

4.2.3. Early and Late responders to ethanol showed G protein expression after repeated exposures to ethanol

While the data shown in the previous section showed some trends, none of the observed changes were statistically significant. It was suspected that changes may be masked by differences in individual behaviour of flies. In the previous chapter, it was demonstrated that two subpopulations of flies with different response to ethanol were separated and referred to as early and late responders (Chapter 3, Figure 3.3).

To investigate whether using early and late responders would produce different response in G protein gene expression, qRT-PCR was carried out as described in the previous section. Parental generation of early and late responders were subjected to the same ethanol treatment as described in Table 4.1. G protein mRNA expression was measured in all three Gα genes (G_i, G_s and G_q).

In the early responders, ethanol exposure induced no change in the acute response in naïve flies (one exposure to ethanol) for all three Gα genes (G_i, G_s and G_q). Ethanol caused an apparent decrease in the basal level of chronically treated flies (two exposures) in the G_i and G_q gene but no change was observed in the G_s gene. There was an apparent increase in the G_i gene and an apparent decrease in the G_q gene in the acute response of chronic flies (three ethanol exposures) (Figure 4.4). None of these changes observed were statistically significantly (Student's T-test, $P>0.05$) different from control naive flies (flies that have never been exposed to ethanol). However, it should be noted that these results are similar to those observed in Figure 4.3 for unselected wild-type flies. Both G_i and G_q genes produced the same response in both wild-type and early responders except G_s gene showed less change in the early responders.

With respect to the Gβ subunits ($G\beta_5$, $G\beta_{13F}$ and $G\beta_{76C}$), ethanol administration to early responders caused an apparent increase in the acute response in naïve flies for $G\beta_5$, no

change in both $G\beta_{13F}$ and $G\beta_{76C}$ subunits. No change was observed in all Gβ subunits when assessed for the basal level in chronically treated flies. When early responders to ethanol were exposed three times, no change was observed in the acute response of chronic flies in the Gβ13F subunit but a decrease was observed in the $G\beta_5$ and $G\beta_{76C}$ subunits (Figure 4.5A). The Gγ genes ($G\gamma_1$ and $G\gamma_{30A}$) were only tested using the early flies. This is because they did not show apparent changes when using the wild-type flies. Ethanol administration did not induce any significant changes in any of these genes. The acute response in naïve flies indicated an apparent increase in both Gγ genes whilst the basal level response in chronic flies showed an apparent increase in the $G\gamma_1$ gene and no change in $G\gamma_{30A}$ gene. There was no change in the acute response in chronic flies for both Gγ genes (Figure 4.5B).

In the late responders to ethanol, the Gα genes tested (G_i, G_s and G_q), no change was observed in the acute response in naïve flies for all three Gα genes. In contrast to the early responders, no change was observed in the basal level of chronic flies in both the G_i and G_s gene but a slight increase in the G_q gene. The G_s gene also showed no change in the acute response in chronic flies compared to the G_i and G_q gene, which showed an apparent decrease relative to control (Figure 4.6). In the Gβ subunits ($G\beta_5$, $G\beta_{13F}$ and $G\beta_{76C}$), no change was observed in the acute response of naïve flies and the basal level in chronic flies relative to control in the $G\beta_5$ and $G\beta_{13F}$ genes except the $G\beta_{76C}$ gene that showed an apparent increase in both responses. There was an apparent increase in the acute response in chronic flies for all Gβ subunits (Figure 4.7).

In summary, while no statistically significant difference was obtained in any of the G protein genes for any of the treatments, the more interesting results appeared to be the changes in G_i and G_q in chronically treated flies. For these proteins, there were similar responses in the wild-type and the early responders but not in the late responders. As it

will be seen in the next chapter, the change in expression of G_i and G_q subunits could indeed be part of the mechanism of tolerance.

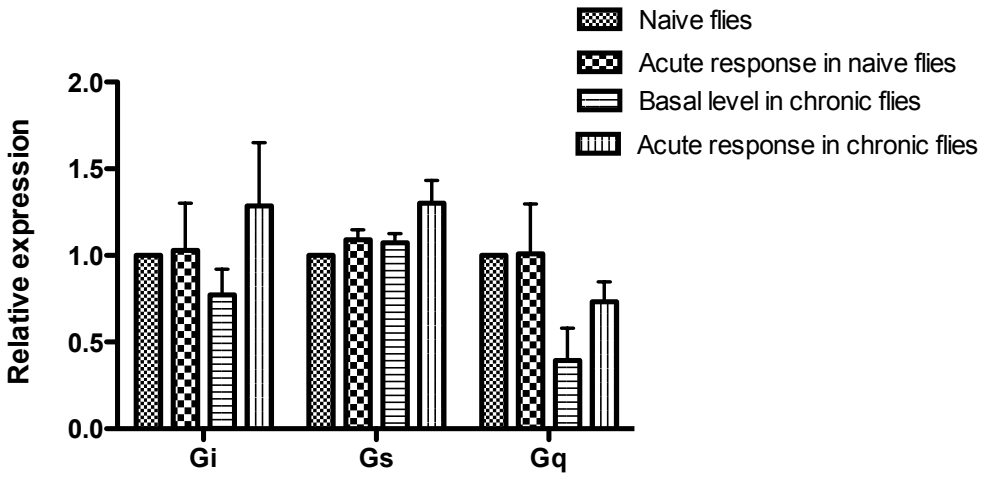

Figure 4.4. Relative Gα gene expression (Gi, Gs, Gq) in the head of early responders

Gene expression of Gα subunits (G_i, G_s and G_q) after flies developed tolerance to ethanol. Early responders were exposed to ethanol once (the acute response in naïve flies), twice (the basal level in chronic flies) and three times (the acute response in chronic flies) were killed 1 h after the last ethanol exposure except for flies exposed twice, which were killed 24 h after the last exposure. Relative expressions to naïve flies (flies that have never been exposed to ethanol) were and normalized to β-actin. Error bars represent ± Standard Error of the Mean (SEM) (Student's T-test, $P<0.05$; n = 5 female flies). Data are representative of three independent experiments.

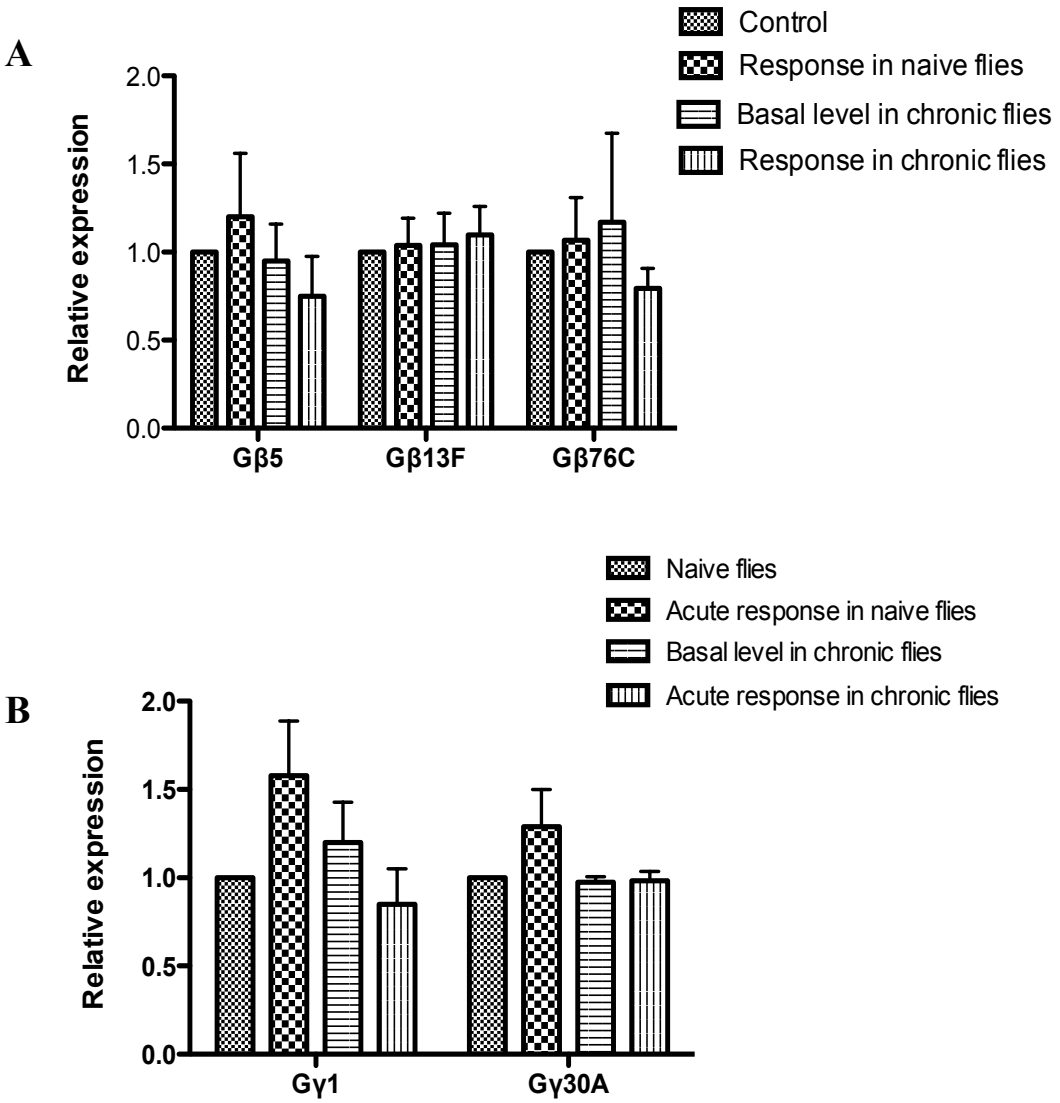

Figure 4.5. Relative Gβγ mRNA expression in the head of early responders

Flies exposed to ethanol once (the acute response in naïve flies), twice (the basal level in chronic flies) and three times (the acute response in chronic flies) were killed 1 h after the last ethanol exposure except flies exposed twice which was 24 h later and subjected to RT-PCR. A) Relative expression of Gβ subunits after three consecutive ethanol exposures (B) demonstrates the gene expression of Gγ subunits. Error bars represent ± SEM (Student's T-test $P<0.05$; n = 5 female flies). Data are representative of three independent experiments.

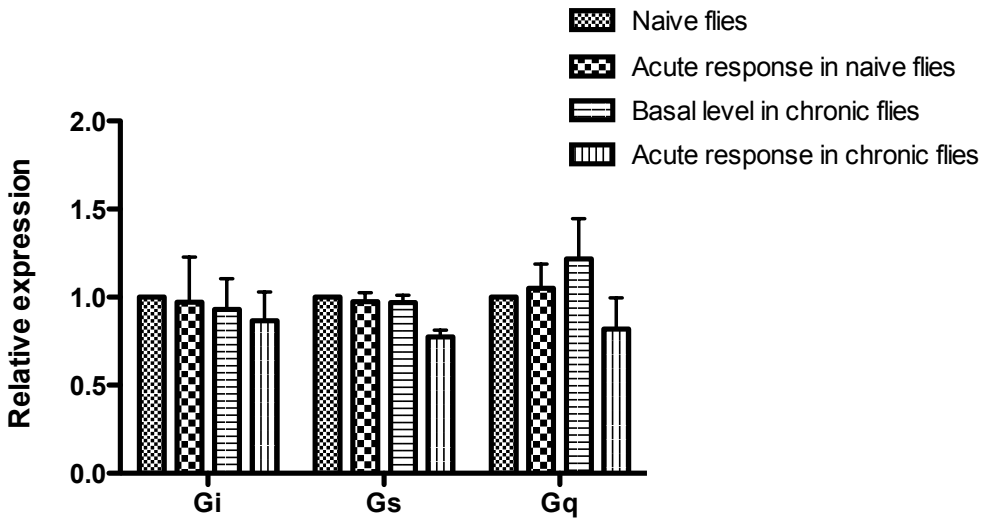

Figure 4.6. Relative Gα gene expression (Gi, Gs and Gq) in the head of late responders

Late responders to ethanol were exposed to ethanol to ethanol once (known as the acute response in naïve flies), flies exposed to ethanol twice (the basal level in chronic flies) and flies exposed to ethanol three times (the acute response in chronic flies) were killed 1 h after the last ethanol exposure except for flies exposed twice, which were killed 24 h after the last exposure. Relative expressions to naïve control flies (flies that have never been exposed to ethanol) were quantified and normalized to β-actin. Error bars represent ± SEM (Student's T-test $P<0.05$; n = 8 female flies). Data are representative of three independent experiments.

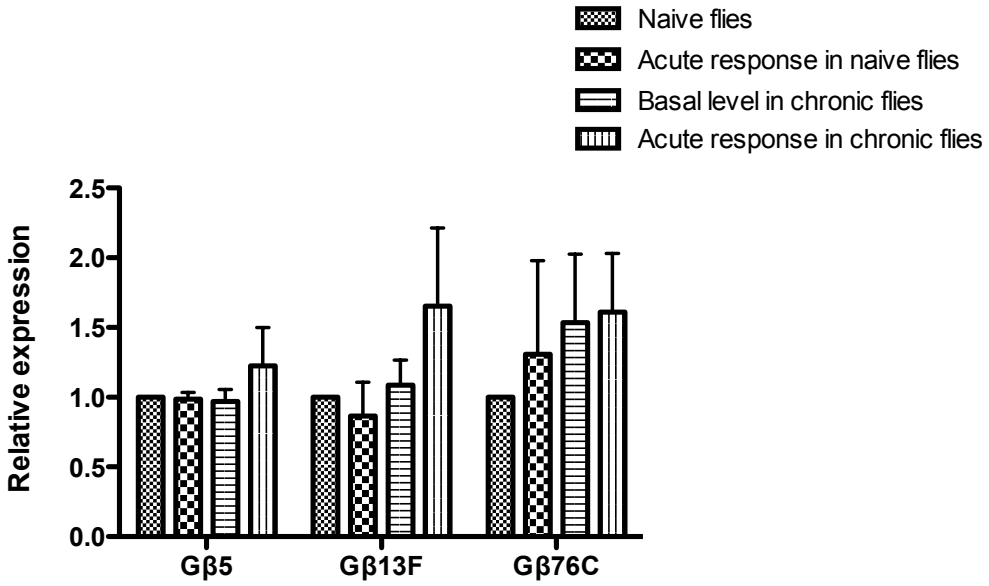

Figure 4.7. Relative Gβ genes expression in the head of late responders

Relative gene expression of Gβ subunits in the pooled head of late responders exposed to ethanol once (acute response in naïve flies), twice (basal level in chronic flies) and three times (acute response in chronic flies). Error bars represent ± SEM (Student's T test $P<0.05$, n = 5 female flies). Data are representative of three independent experiments.

4.2.4. Western blot

Ethanol-induced gene expression of some G protein genes suggest that specific G protein subunits may play a role in alcohol addiction processes, including tolerance. To characterise the expression of these subunits at the protein level, western experiments were carried out to determine the effect of alcohol on changes in G proteins level as described in Section 2.6.5. Optimisation experiments were carried out to determine the concentration of antibodies using a dot blot (Figure 4.8). This involved pipetting 2µl of different dilutions (1:50, 1:100, 1:200 and 1:500) of primary antibody (G_q antibody) onto a nitrocellulose paper. The membrane was then blocked and incubated with the appropriate secondary antibody. 1:500 dilution was chosen for G_q antibody due to its high signal.

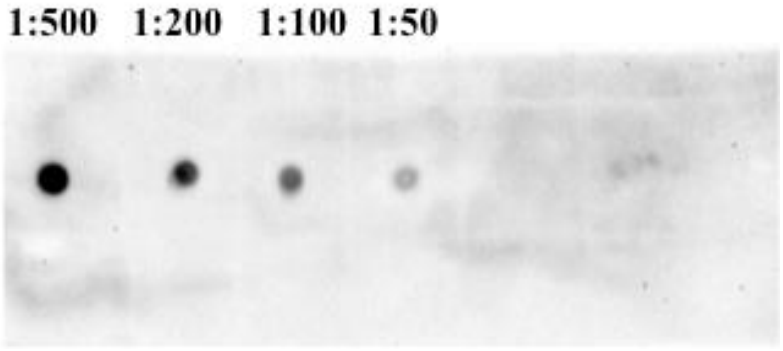

Figure 4.8. Dot blot of G_q antibody

Different concentration of G_q antibody (1:50, 1:100, 1:200 and 1:500) was placed onto a nitrocellulose paper, blocked and then incubated with secondary antibody.

To optimise the assay, different volumes of protein samples (2µg, 5µg and 10µg) extracted from the head of wild-type flies and brain tissue of mice (used as a control) were loaded on a 10% prepared SDS-PAGE gel. Proteins were detected with anti G_q and β-actin antibodies (Figure 4.9). Non-specific bands were observed using G_q

antibody in Figure 4.9B, this could be due to protein aggregation that is not resolved by boiling or the secondary antibody. Approximate weights are shown on the left.

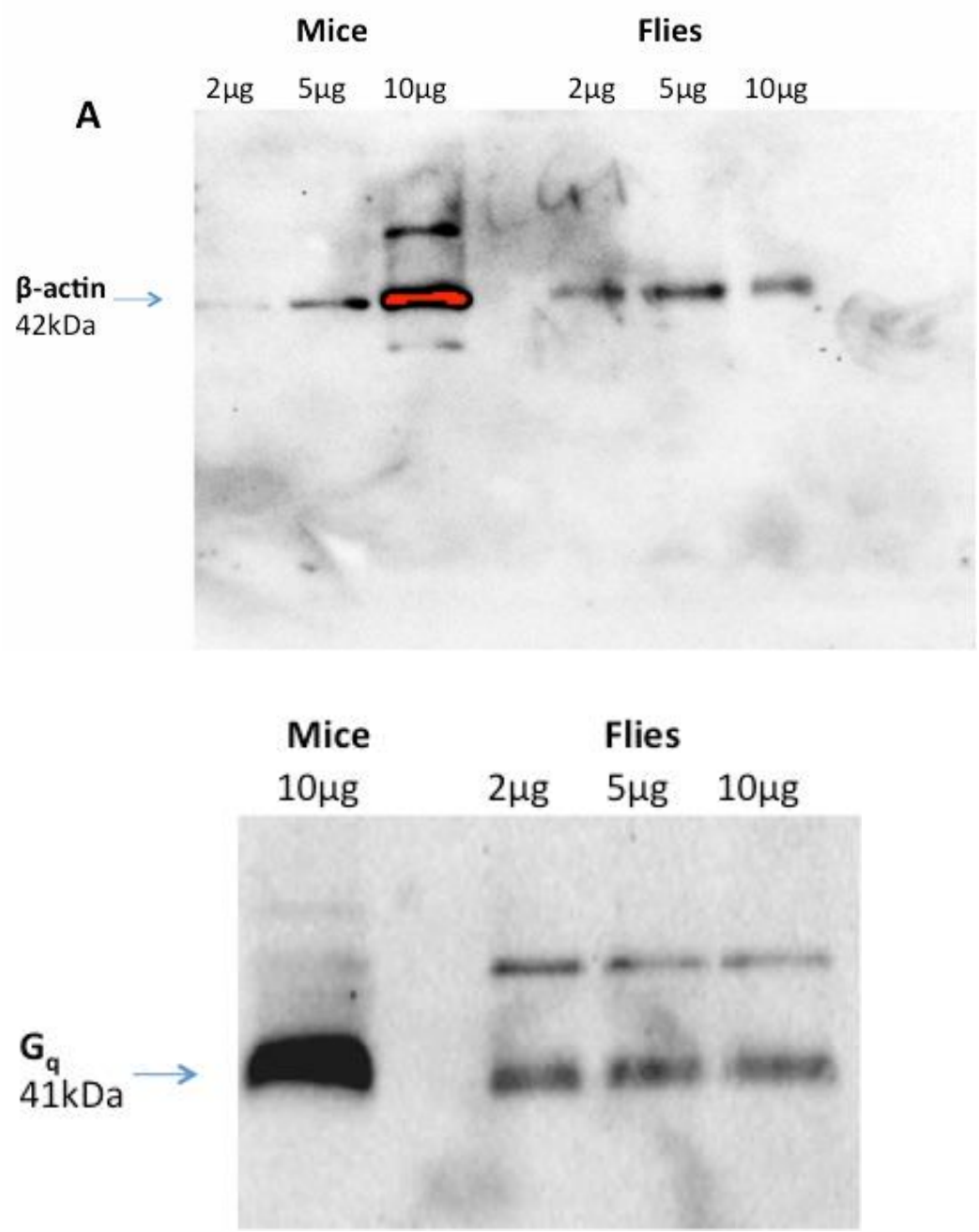

Figure 4.9. Western blots of mouse brain tissue and fly head.

Different amounts of protein samples were loaded into each well for both mice (used as a control) and flies stained with (A) β-actin (1:1000 dilution) and (B) G_q (1:500 dilution). Numbers on the top of the blot are the amount of lysate loaded to each well. (n= 5 fly heads).

Protein samples were extracted from fly heads and mice brain and analysed by western blot. Initial experiments were promising for G_q but no signal was even detected with G_i antibody in the fly or mice sample (Figure 4.10D). Unfortunately, the G_q antibody proved to be unstable (Figure 4.10C). Despite purchasing new batches of antibodies, the problem was unsolved. Other investigators have discussed issues online regarding antibodies from the company (http://www.ihcworld.com/smf/index.php?topic=1986.0) and the company in question discontinued the product when contacted. The project could not be continued.

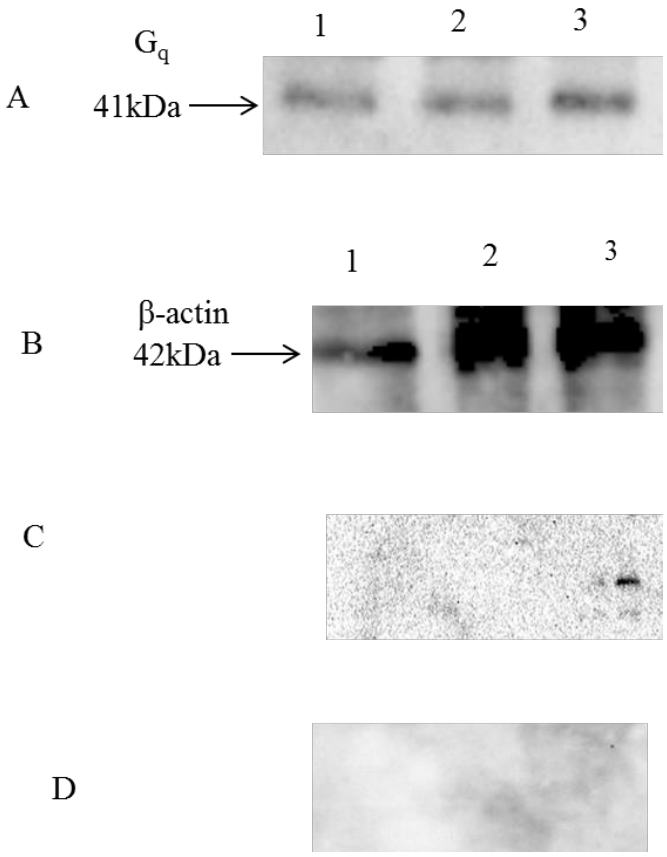

Figure 4.10. Western blot analysis of alcohol treated flies.

Equal amounts of protein samples of ethanol exposed female flies and naïve flies. Equal amounts of sample (5µg) were used for western blot analysis of one ethanol exposure (n = 5), three ethanol exposures (n = 5) using 1: 500 dilution of G_q antibody (A) and 1:1000 dilution of β-actin antibody (B). Representative blots showing the lack of function using (C) G_q and (D) G_i antibodies.

4.3. Discussion

Previous work by other investigators has established *Drosophila* as a useful model for the identification of genes that respond to ethanol exposure (Moore *et al*., 1998, Kaun *et al*., 2012). Other studies have previously suggested the correlation of abused drugs and G protein subunits (reviewed by Kitanaka *et al*., 2008). Nestler and colleagues reported some changes in G proteins subunits in specific brain regions of the rat locus coeruleus following chronic morphine administration that produces states of tolerance and dependence (Nestler *et al*., 1989).

It was reported that expression levels of G protein subunits after chronic morphine administration caused an up-regulation of G_i subunit (Nestler *et al*., 1989). They hypothesized that increased expression of G_i subunits may reduce the possibility of association between Gβγ dimers and G_s subunit, resulting in increased free G_s subunit, which can activate adenylyl cyclase (Nestler *et al*., 1989). Other studies have also reported that a single morphine injection causes an increase in the Gα and Gβ subunits in rat pineal glands (Chetsawang *et al*., 1999); acute and chronic treatment induced gene expression changes in some G protein subunits (G_o, G_{i1}, G_{i2} and $G\beta_1$) (Kaewsuk *et al*., 2001); mRNA levels of $G_{i/o}$ and G_s were changed in rats after morphine-induced CPP (Zelek-Molik *et al*., 2012).

There have been fewer studies in the literature on the effect of ethanol on G proteins (Chapter 1, Section 1.3.3). To the best of the author's knowledge, this study provides the first demonstration of the measurement of G protein expression levels after ethanol tolerance in *Drosophila*, to show if there is a contribution to addictive processes.

In this study, quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure the relative expression of G protein subunits in *Drosophila's* head. This methodology is particularly suited for quantifying the expression of genes with

homologous sequences as it exploits the high sensitivity and specificity of real-time polymerase chain reaction (Schmittgen and Livak, 2008).

The relative expression of G protein subunits was measured in the head of *Drosophila* following ethanol exposure as a method for studying gene expression. Measuring relative expression in mRNA levels is a convenient way to investigate whether molecular changes are occurring, but further studies are required to determine the mechanisms of the changes of mRNA levels and whether they result in changes of protein levels and changes in physiological function. Our data suggests that there are changes in the expression of G protein subunit levels after wild-type flies have been exposed to ethanol one, two and repeatedly for three days (shown in the previous chapter to induce behavioural tolerance).

The initial screening of G protein gene expression changes with response to ethanol, was carried out on a wild-type population of *Drosophila*. While gene expression changes in the ethanol exposed flies were observed, the results were not easy to reproduce when multiple experiments were pooled and the results were non-statistically significant. This suggested that there were potential genetic variations within the wild-type population and indeed, the behavioural experiments presented in Chapter 3 indicated that some flies may respond early to ethanol while some may respond late to ethanol. In the subpopulations of *Drosophila* (early and late responders to ethanol) that were selected for high ethanol sensitivity, there was a non-statistically significant decrease in G_i and G_q genes. It was unfortunate that the changes in gene expression could not be confirmed at the protein level due to unsatisfactory performance of the available commercial antibodies.

One reason for differences between the wild-type population and the subpopulation could be that the flies respond differently to the sedating effects of ethanol, which may lead to different results with respect to G protein gene expression. The most obvious

changes in G protein gene expression were observed in the early flies. Therefore, it could be that flies that have a more sensitive response to ethanol induce clearer changes in gene expression. This could be due to the fact that a threshold needs to be overcome for the effect to be triggered or that late responders flies have a compensating mechanism. An alternative explanation for the variability between results obtained, maybe a larger number of flies may have been required to obtain statistically significant changes or the number of experiments was too small.

Observed changes in the expression of G proteins could have an important physiological role. The prefrontal cortex receives innervations from the ventral tegmental area, which is implicated in reward behaviour (Chao and Nestler, 2004). The decreased expression of G_i for example, induced here by ethanol exposure could lead to a decrease in the number of G-coupled receptors associated with this G protein subunit at resting state and a parallel increase of G protein coupled receptors associated to other G protein subunits (Kaewsuk *et al*., 2001). Upon receptor stimulation, in flies treated with ethanol, a lower number of G_i subunit and a greater number of other subtypes of G protein subunits would be released and a different population of specific effectors would be activated.

4.4. Conclusion

In this chapter, G protein gene expression changes were observed in the head of *Drosophila* following tolerance. The findings presented in this chapter, demonstrated some apparent changes in some G protein subunits after ethanol tolerance in both wild-type and in a selected subpopulation of early and late responders to ethanol. Wild-type flies showed greater variations within the expression levels in mRNA levels compared to the subpopulation of selected flies, which showed less variations but a non-significant decrease in some genes. The next chapter will demonstrate statistically significant changes in the gene expression of selected G protein subunits in a more homogenous population of *Drosophila* mutants such as G protein mutants and dopamine 1-like receptor 2 mutants.

Chapter 5: G protein gene expression changes in *Drosophila* mutants

Summary

This chapter extends the work described in Chapter 4 by measuring the effect of alcohol on G protein expression in *Drosophila* lines that carry mutations in either a G protein gene or the dopamine D1-like receptor. The aim is to validate the observations described in Chapter 4 and to attempt to understand the behavioural significance and mechanism of action of the observed changes. Section 5.1 provides a brief introduction to the study and describes the need for these experiments. Section 5.2 provides an analysis of the results while 5.3 is the discussion and 5.4 the conclusion of this part of the investigation.

5.1. Introduction

Ethanol administration affects signalling in the central nervous system and affects the modulation of gene expression in the brain. Alterations in neuronal structure, biochemistry and function have been considered the reason behind the initiation and maintenance of drug addiction and dependence (Miguel-Hidalgo, 2009). For example, neuroadaptive changes that occur in the mesocorticolimbic dopaminergic system, a reward centre of the brain are thought to underline the process of alcohol tolerance and dependence (Flatscher-Bader *et al*., 2006). Indeed, changes in gene expression can indicate enduring adaptations in the brain, thereby leading to the unchangeable progression from controlled to compulsive ethanol use (Spanagel, 2009). This molecular approach has been very successful in providing very rich knowledge on the neuronal pathways and brain circuits that are altered in response to drugs and on how neuronal alterations modulate specific abnormal behaviours (Miguel-Hidalgo, 2009).

Several research groups have designed experiments to identify phenotypes that may be associated with alcohol addiction. The *Drosophila* model offers powerful genetic and molecular methodologies with which to dissect genes and to study complex trait disorders such as alcoholism and to examine the effects of ethanol (Awofala, 2011; Berger *et al*., 2008). *Drosophila* have well-understood genetics with publicly available collections of mutation at single loci (Bellen *et al*., 2004). *Drosophila* mutants have been used to study susceptibility to the effects of ethanol by measuring the level of sensitivity and tolerance to the sedating effects of ethanol (Corl *et al*., 2009; Cowmeadow *et al*., 2006; Moore *et al*., 1998; Rothenfluh *et al*., 2006; Scholz *et al*., 2000). *Drosophila* mutants have thus become a useful tool to understand the molecular processes involved in the manifestation of tolerance that could be shared with mammals.

In the previous chapter, in the wild-type and subpopulations (early and late responders) of *Drosophila*, gene expression of the Gα subunits, including G_s, G_i, and G_q, were detected by quantitative real-time polymerase chain reaction (qRT-PCR) in the fly heads after recurrent exposures to ethanol. The subunits, G_s, G_i, and G_q, showed the most reproducible change as compared to other subunits such as G_s that remain predominately unchanged. This suggests that these subunits could be involved in the molecular pathways regulating the behavioural response to ethanol's effects and provide insights into the adaptive and pathological mechanisms within the nervous system (Chapter 4). To further explore the observed ethanol-induced changes in G protein gene expression, it was decided to exploit the availability of *Drosophila* mutants that can be obtained from *Drosophila* banks (e.g. flybase.org). The *Drosophila* lines that were selected carry mutations in two G proteins genes and in the Dopamine D2 receptor (Table 5.1). Their details are described below.

Table 5.1. Shows the list of mutants used. These *Drosophila* strains were selected and purchased from the *Drosophila* Stock Centre (Bloomington, IN, US).

Bloomington Stock number	Annotated symbol	Affected gene	Genotypes	Notes
30736	CG17759	G_q	Gαq1370/CyO	Reduced activity of G_q protein function by point mutation (Kain *et al*., 2008)
17672	CG10060	G_i	y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}Gal phai[EY10355]	Disruption of G_i protein function by P-element insertion (Bellen *et al*., 2004)
24743	CG18741	Dopamine 1-like receptor 2 (Dop1R2)	w1118; Mi{ET1}Dop1R2MB05 108	Disruption of dopamine D2 function by *Minos* insertion (Metaxakis *et al*., 2005)

5.1.1. G protein subunit gene mutants

The G proteins are a family of proteins that relay signals from G protein-coupled receptors to effector molecules and are composed of three subunits, α, β, and γ (Boto *et al*., 2010; Downes and Gautam, 1999). Two of the genes in Gα subunits (G_q and G_i) showed some changes in gene expression in the previous chapter and will be studied further in this chapter.

The G_q gene also known as $G\alpha_q$, is often expressed ubiquitously and its most common pathway in *Drosophila*, involves the activation of phospholipase C (PLC), which breaks down phosphatidylinositol 4, 5-biphosphate (PIP_2) into inositol, 4, 5-triphosphate (IP3) and diacylglycerol (DAG). IP3 induces the release of calcium from the smooth endoplasmic reticulum, which work together with DAG to activate protein kinase C (PKC) (Wettschureck and Offermanns, 2005; Wirotanseng *et al*., 2013). The G_q signalling mechanism is essential in the nervous system, however, it has not previously been associated with the ethanol response in *Drosophila*. The mutation of this gene

located on 49B on chromosome 2 was introduced by a commonly used mutagen, ethyl methane sulfonate (EMS) which cause single base changes (point mutation), potentially leading to the reduction of the protein activity by causing missense or non-sense mutation (Kain *et al*., 2008). The G_q mutants produce a transcript with a G to A mutation at base pair 1933. This would produce a change from arginine to lysine at residue 207 (R207K), which lies in the switch II helix regions that is highly conserved among the α subunits of all trimeric G proteins (Kain et al., 2008). The mutation in the G_q gene destabilizes the GTP-bound state of Gq and renders it ineffective in the activation of the downstream effector molecules (Kain *et al*., 2008). The full genotype of the G_q mutant (flybase strain: 30736) is Gαq1370/CyO. CyO is a balancer that gives a curly winged phenotype to the flies which also aids in ensuring that the mutation is maintained in future generations. The genomic structure of the G_q allele is shown below.

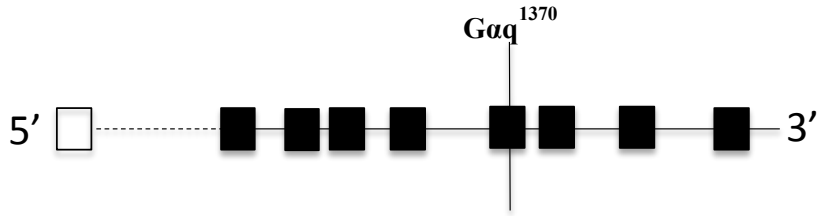

Figure 5.1. Genomic structure of G_q allele

The intron-exon structure is shown with the point mutation of $Gαq^{1370}$ of transcripts G-A at the 5th exon. Exons are designated in rectangles with non-coding exons are in open white box. Nucleotide substitution: G1933A. Protein change 'Amino acid replacement: R207K. Diagram redrawn from flybase.com and National Centre for Biotechnology Information (NCBI).

The G_i gene also known as $Gα_i$ is located on 65A on chromosome 3. It is expressed in several tissues and is known for its role in the inhibition of adenylyl cyclase and thus causes a decrease of cAMP concentration in the cell (Defer *et al*., 2000). The $Gα_i$ gene has been implicated in different types of signalling cascades such as the tyrosine-protein

kinase/mitogen-activated protein kinase and it is also known to inhibit calmodulin activity (Defer *et al*., 2000). It is also been reported to play a role in the activation of the phospholipase C (PLC) pathway (Mizuta *et al*., 2011). The mutation in the G_i gene, $G\alpha_i^{EY10355}$, in the *Drosophila* that was used in this study was engineered by a P-element insertion, which potentially disrupts the gene function (Bellen *et al*., 2004). The G_i gene has not been previously implicated in ethanol response in *Drosophila*.

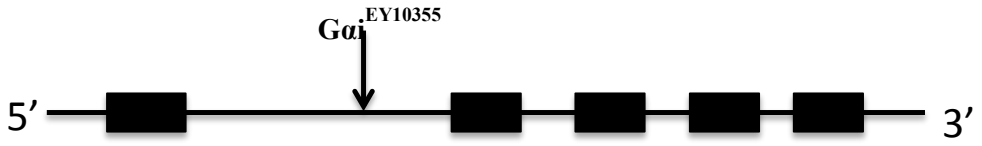

Figure 5.2. Genomic structure of G_i allele

The intron-exon structure is shown with the P-element insertion on the 1st intron position, which spans ~ 5044bp. Exons are designated in rectangles. Diagram redrawn from flybase.com and NCBI.

5.1.2. Dopamine receptor mutant

Ethanol exerts its effects through multiple neurotransmitter systems including dopamine, GABA and serotonin systems. Dopamine signalling is crucial for the actions of ethanol in *Drosophila* and it has been implicated in brain mechanisms of reward, reinforcement and addiction (Diamond and Gordon, 1997). Disruption or deletion of dopamine signalling in *Drosophila* results in decreased locomotor hyperactivity and prevents the stimulatory effects of most drugs of abuse including cocaine (Bainton *et al*., 2000; Li *et al*., 2000).

The *Drosophila* dopamine 1-like D2 receptor (Dop1R2) belongs to the dopamine receptor family and has a high affinity for dopamine with amino acid sequences homologous to those of mammalian D1-like receptors. D1-like dopamine receptors bind to the stimulatory G protein (G_s), thus initiating the activation of adenylyl cyclase

(Hearn *et al*., 2002; Vickrey and Venton, 2011). The mutation in the Dop1R2 is generated by *Minos* insertion, which led to the disruption of the Dop1R2 function and carries the balancer TM3 that carries the phenotype 'stubble' (Metaxakis *et al*., 2005). Mutants of Dop1R2 have been found to be associated with the ethanol tolerance response (Kong *et al*., 2010).

Figure 5.3. Genomic structure of Dop1R2 allele

The intron-exon structure is shown with the *Minos* insertion in the 3rd intron, which spans ~ 1636bp. Mutants were produced from the transcripts C-A on the same region. Exons are designated in rectangles. Diagram re-drawn from flybase.com and NCBI.

5.1.3. Characterisation of *Drosophila* mutants

The mutant strains were obtained from the reputable Bloomington *Drosophila* Stock centre. Two of the lines (G_q and Dop1R2) carried balancer, which aids in ensuring that the mutations are maintained in the specific lines. To ensure the authenticity of the mutant strains, further tests could be carried. Point mutations can be verified by amplifying and sequencing the mutated genes, and the presence and location of transposon could be checked by amplifying the genomic sequence flanking the reporter insertion site. These latter tests were not carried out in this project. In retrospect, it would have been preferable to do so.

5.1.4. Objective

In the previous chapters, using the wild-type and subpopulations (early and late responders) of *Drosophila*, some G protein subunits whose expression was altered by ethanol administration were identified. Although the observed G protein changes did

not reach a statistical significance, it was reasoned that this might be due to genetic variation within the wild-type population, which was confirmed by the more consistent results, obtained in the behaviourally selected early and late flies. Thus using a more homogenous population such as those selected for specific mutations, was expected to produce more reproducible results and would allow to explore the mechanisms induced by ethanol exposure.

The specific objectives of this chapter were:

- To measure the effect of mutations of G_q, G_i and Dop1R2 genes on ethanol-induced behaviour.
- To measure the effect these mutations will have on ethanol induced G protein gene expression.

5.2. Results

5.2.1. Ethanol-induced behaviour and gene expression in G_q mutants

5.2.1.1. Effect of ethanol on the behaviour of G_q mutants

In order to investigate what effect mutations in the G_q gene have on ethanol-induced behaviour, female G_q mutants and wild-type flies (used as control) were exposed to ethanol repeatedly for three days allowing 24h recovery period to test if they can acquire tolerance to the sedating effects of ethanol (Protocol shown in Figure 5.4A). During the first few minutes of ethanol exposure, the mutant flies entered a hyperactivity state, in which they walked more, vibrated their wings and climbed the walls of the vial at greater speed (Maples and Rothenfluh, 2011). In all cases, the time to 50% sedation (ST50) of the first, second and third exposure was determined (Maples and Rothenfluh, 2011). Ethanol tolerance was quantified as an increase in ST50 caused by prior ethanol exposure.

The G_q mutants showed a significantly higher ST50 in all ethanol treatments compared with the wild-type flies (Figure 5.4B). A significant increase was only observed between the first and third exposures but not between the second and third exposures. This finding suggests that G_q mutants are less sensitive to the sedating effects of ethanol compared to the wild-type population. To the author's knowledge, this is the first report demonstrating ethanol-induced behaviour in G_q mutants.

5.2.2. The effect of ethanol on G protein gene expression in G_q mutants

To examine whether mutation in the G_q gene will have an effect on ethanol-induced expression, selected G protein subunits (G_i, G_s and G_q) were tested. As described in the previous chapter (Chapter 4, Table 4.1), acute response in naïve flies (one ethanol treatment), basal level in chronic flies (two ethanol treatments) and acute response in

chronic flies (three ethanol treatments) was quantified and analysed using quantitative real-time polymerase chain reaction (qRT-PCR) in G_q mutants compared with naïve flies (untreated flies).

The relative G protein expression in the head of G_q mutants indicates that consecutive ethanol treatments did not induce significant changes in both the G_i and G_s subunit compared with naïve flies (Figure 5.5). In contrast, ethanol treatments induced significant changes in the relative expression in the G_q subunit. A slight increase was observed in the acute response in naïve flies, a significant decrease ($P<0.05$) in the basal level in chronic flies and a further significant decrease ($P<0.01$) in acute response in chronic flies. Ethanol-induced treatment did not cause any significant change in the G_s subunit (Figure 5.5).

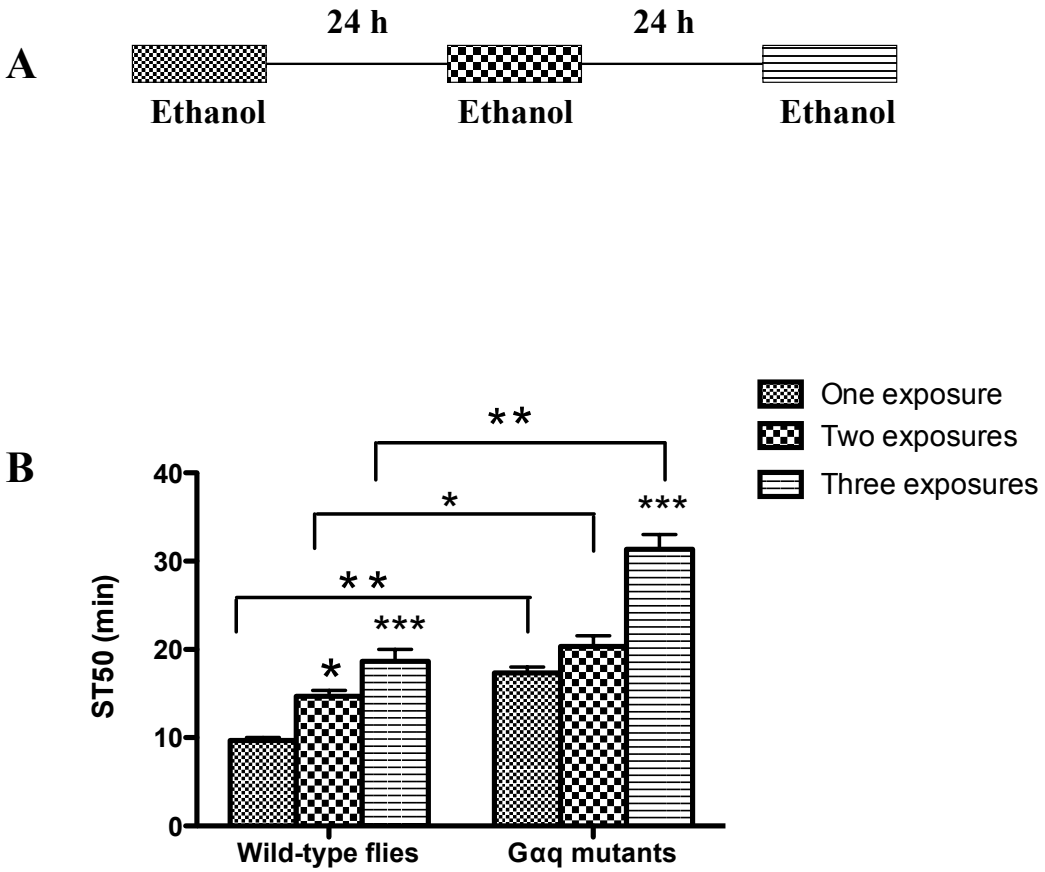

Figure 5.4. Tolerance induction in $G\alpha_q$ mutants

(A) Ethanol treatment regime used to induce and measure ethanol sensitivity and tolerance. Eight female flies were initially exposed to 500µl of 100% ethanol to measure the sensitivity of flies to the sedating effects of ethanol. The time to 50% sedation (ST50) – the time it takes for half of the ethanol exposed flies to become stationary – was used as a measurement of sensitivity to ethanol. To measure tolerance, flies were exposed to the same dose of ethanol 24h later for 2 consecutive days. (B) G_q mutants have a point mutation that results in an inactive G_q protein. G_q mutant flies are less sensitive to the sedating effects of ethanol compared to the wild-type control in the sensitivity assay. The G_q mutants displayed a significant increase in ST50 in all treatments compared to wild-type flies (n= 8 female flies in each group). Error bars represent the standard error of the mean (SEM) and asterisk denotes statistical significance by Two-way analysis of variance (ANOVA). *P<0.05, **P<0.01, ***P<0.001 (n=3 independent experiments).

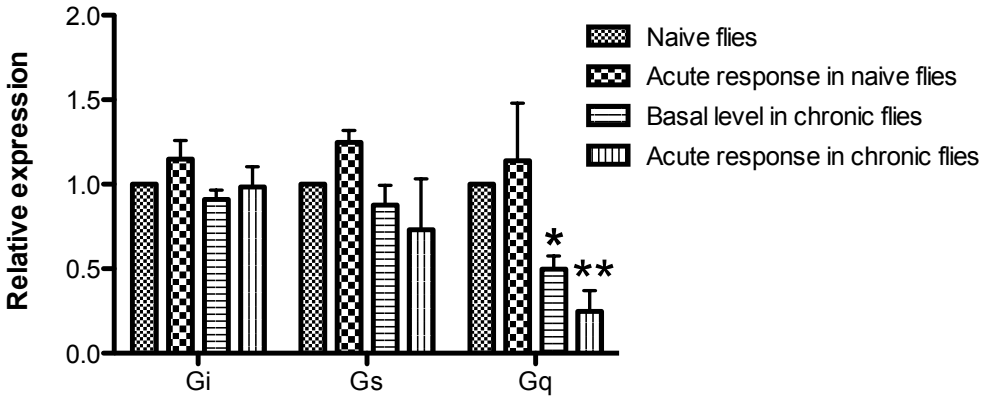

Figure 5.5. Relative Gα genes expression in the head of G_q mutants

Gα genes expression in the head of G_q mutants were measured after different ethanol treatments. G_q mutants were exposed once, twice or three times to 500µl of 100% ethanol. The flies were killed 1h after the first (acute response in naïve flies), last (acute response in chronic flies) and 24h after the second exposure (basal level in chronic flies) and compared to naïve flies (never exposed to ethanol). Relative expressions were quantified using $2^{\Delta\Delta Ct}$ method and normalized to the housekeeping gene, β-actin relative to untreated control. Error bars represent ± SEM. Asterisk denotes statistical significance by Student's T-test **P<0.01 (n = 5 female flies). Data are representative of three independent experiments.

5.2.3. Ethanol-induced behaviour and gene expression in G_i mutants

5.2.3.1. Effect of ethanol on the behaviour of G_i mutants

To assess the effect mutation of the G_i protein has on ethanol-induced behaviour, female G_i mutants were analysed and compared with wild-type flies to test if they acquire tolerance to ethanol. G_i mutants displayed significantly higher ST50 compared with wild-type flies in all ethanol treatments (one, two or three) (Figure 5.6). This result reveals that G_i mutants can develop tolerance but are less sensitive to ethanol's effects than wild-type flies. To the author's knowledge, this is the first report of a difference in ethanol-induced behaviour in these G_i mutants.

5.2.3.2. The effect of ethanol on G protein gene expression in flies mutated in G_i gene

To investigate whether mutations in the G_i gene will alter ethanol-induced G-protein gene expression, flies sacrificed one hour after one ethanol exposure (acute response in naïve flies), 24h after two ethanol exposure (basal level in chronic flies) and 1h after three ethanol exposures (acute response in chronic flies) were quantified and analysed compared with naïve flies (untreated).

Expression of G_s, G_q and G_i was detected in the flies. Detecting any G_i expression of these mutants was not expected and is discussed below. Ethanol treatment induced significant changes in both the G_i and G_q subunits. In the G_i subunit, there was a significant increase ($P<0.05$) observed in the acute response in naïve flies but a significant decrease ($P<0.01$) in the basal level and acute response in chronic flies ($P<0.05$) compared with naive flies. In the G_q subunit, an apparent decrease ($P>0.05$) was observed in the basal level in chronic flies but significant decrease ($P<0.05$) was observed in the acute response in chronic flies. Ethanol treatment induced an apparent

increase in the acute response in naïve flies for the G_s subunit and no change in both the basal level and acute response of chronically treated flies (Figure 5.7).

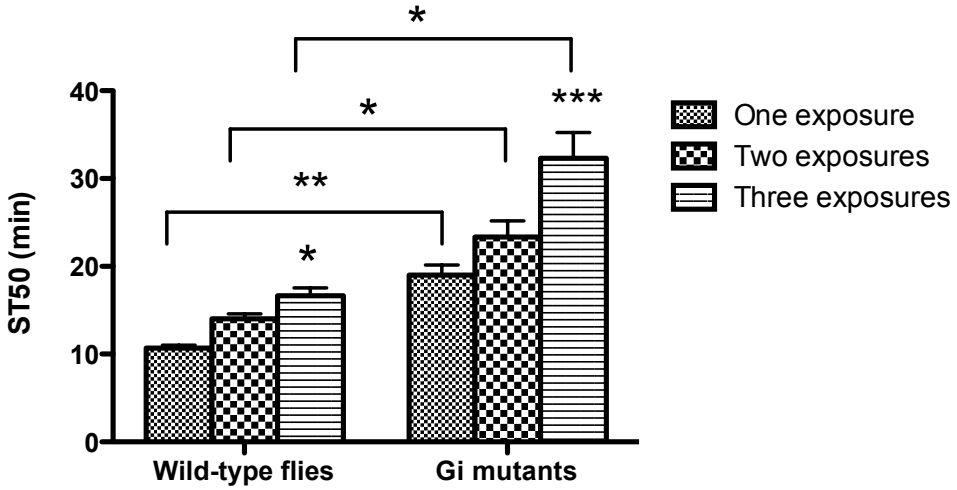

Figure 5.6. Tolerance induction in G_i mutants

G_i mutants have disruption of the G_i protein. $G\alpha_i$ mutant flies are less sensitive to the sedating effects of ethanol compared to the wild-type control in the sensitivity assay. The G_i mutants displayed a significantly higher ST50 in all exposures compared to wild-type flies (n = 8 female flies in each group). Error bars represent the standard error of the mean (SEM) and asterisk denotes statistical significance by Two-way ANOVA. *P<0.05, **P<0.01, ***P<0.001 (n=3 independent experiments).

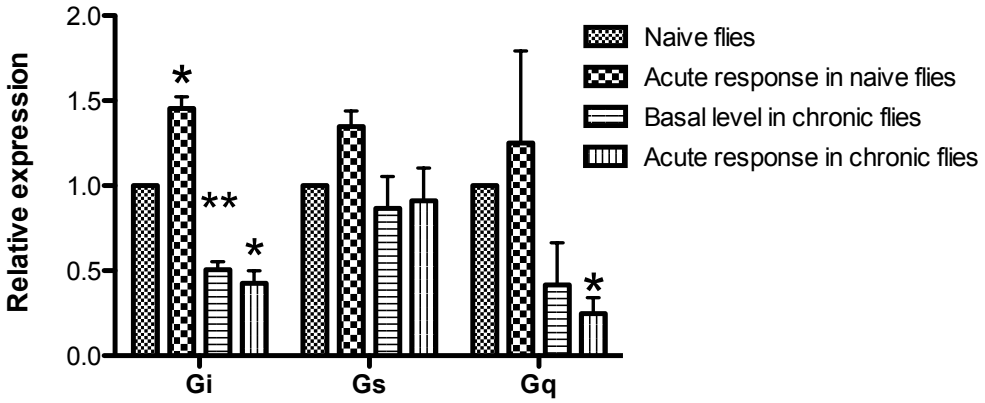

Figure 5.7. Relative Gα genes expression in the head of G_i mutants

Gα genes are expressed in the head of G_i mutants after different ethanol treatments. G_i mutants were exposed one, two and three times to ethanol. The flies were killed 1h after the last ethanol exposure except for flies exposed twice, which were killed 24h after the last exposure. Relative expressions to naïve flies were quantified normalized to the housekeeping gene, β-actin relative to naïve control flies. Error bars represent ± SEM. Asterisk denotes statistical significance by Student's T-test, **P<0.01 (n = 5 female flies in each group). Data are representative of three independent experiments.

5.2.4. Ethanol-induced behaviour and gene expression in Dopamine 1-like receptor 2 (Dop1R2) mutants

5.2.5. Ethanol-induced behaviour in the Dop1R2 mutants

Dop1R2 mutants displayed lower ST50 in all ethanol treatments compared with wild-type *Drosophila* (Figure 5.8). Between each treatment, Dop1R2 showed significant increase in ST50. This implies that the Dop1R2 mutant have higher sensitivity to the sedating effects of ethanol and have the ability to develop tolerance following repeated ethanol exposures.

5.2.6. The effect of ethanol on G protein gene expression in Dop1R2 mutants

To investigate whether mutations in the Dop1R2 have an effect on ethanol-induced G protein gene expression in G_i, G_s and G_q subunits, mutated flies were subjected to the same ethanol treatment and sacrificed as explained previously.

Ethanol treatments caused significant changes in some of the subunits. One ethanol treatment (acute response in naïve flies) caused an apparent decrease in all three subunits. The two-ethanol treatments (basal level in chronic flies) induced an apparent decrease ($P>0.05$) in the G_i subunit and a significant decrease ($P<0.05$) in the G_q subunit. Significant decrease ($P<0.001$) after three ethanol treatments (acute response in chronic flies) was observed in both G_i and G_q subunits. No significant change was observed in the G_s gene for all ethanol treatments (Figure 5.9).

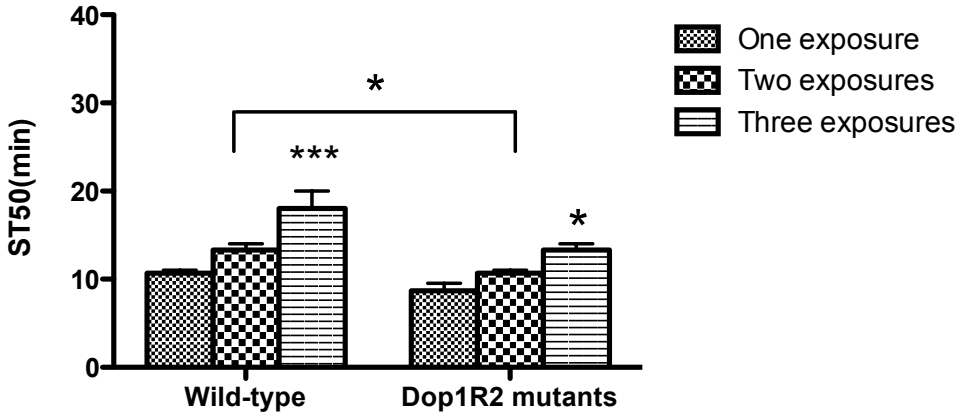

Figure 5.8. Tolerance induction in Dop1R2 mutants

Dop1R2 mutants do not express functional dopamine 1-like receptor 2. The time to 50% sedation (ST50) – the time it takes for half of the ethanol exposed flies to become stationary – was used as a measurement of sensitivity to ethanol. Dop1R2 flies are more sensitive to the sedating effects of ethanol compared to the wild-type control in the sensitivity assay, which is displayed as a reduced response in ST50 (n = 8 female flies in each group). Error bars represent the SEM and asterisk denotes statistical significance by Two-way ANOVA. *P<0.05, **P<0.001. (n=3 independent experiments).

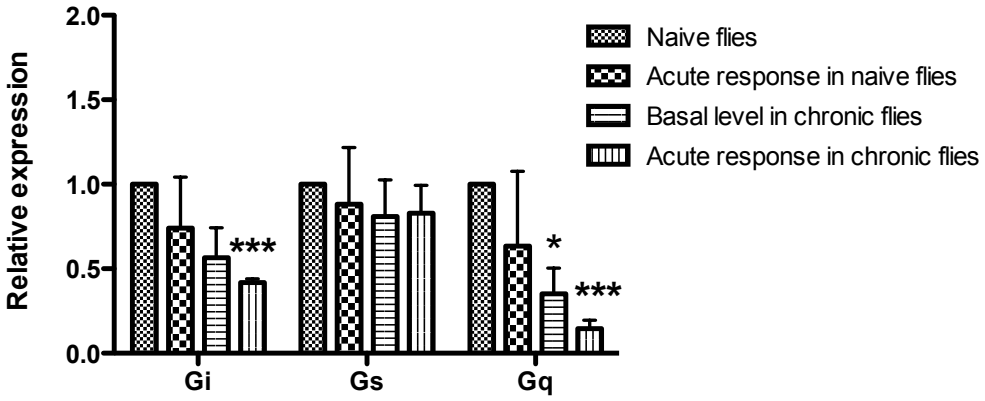

Figure 5.9. Relative Gα expression in the head of Dop1R2 mutants

Gα genes expression in the head of Dop1R2 mutants after different ethanol treatments. Dop1R2 mutants were exposed to ethanol one (acute response in naïve flies), two (basal level in chronic flies) and three (acute response in chronic flies) times and killed 1 h after last exposure except for the two times exposure which was 24 h. Relative mRNA expression to naïve flies were quantified for G_i, G_s and G_q. Error bars represent ± SEM. Asterisk denotes statistical significance by Student's T-test, **P<0.01 (n = 5 female flies). Data are representative of three independent experiments.

5.3. Discussion

In the previous chapter (Chapter 4), it was demonstrated that G_q subunit showed a decrease in gene expression in the basal level of chronically treated flies and acute response of chronic flies when studied in the wild-type and subpopulation of *Drosophila*. To understand further the significance of the observed ethanol-induced changes in G proteins, *Drosophila* strains carrying mutation in either the G protein gene or in the addiction associated D1-like receptors, were selected and exposed to the same ethanol treatment used for the wild-type populations described in Chapter 4. In the analysis of the data, it is worth nothing that the mutation of G_q gene is a point mutation that affects protein functionality, while the G_i gene and D1-like receptor are disrupted by a large insertion (Table 5.1). Although changes in mRNA gene expression do not necessarily mirror the changes in protein levels, they are widely regarded as indices of alterations of physiological function (Kaewsuk *et al*., 2001; Zelek-Molik *et al*., 2012). Here, the effect of ethanol treatments on G protein subunits (G_i, G_s and G_q) expression in mutant *Drosophila* will be discussed.

5.3.1. Behavioural Differences among mutants

To identify behavioural differences among *Drosophila* mutants compared with wild-type flies, the onset of tolerance was studied in the different strains. The findings (Figures 5.4 & 5.6) indicated that following repeated ethanol treatment, G_q and G_i mutants developed tolerance to the sedating effects of ethanol despite the fact they may not express functional G_q and G_i subunits respectively. The data also showed that both strains had lower sensitivity to alcohol both before and after onset of tolerance (higher ST50). This decrease in ethanol sensitivity in both mutants may allow them to consume more ethanol compared with the wild-type flies.

The Dop1R2 mutants showed a different effect following repeated ethanol treatment. The finding indicated that they had higher ethanol sensitivity both before and after the onset of tolerance (Figure 5.8). The increase in sensitivity in Dop1R2 mutants may allow them consume less ethanol compared to the wild-type *Drosophila*.

5.3.2. Gene expression differences among *Drosophila* mutants

To understand if G protein subunits played a role in the ethanol-induced expression, the expression of G_q, G_i and G_s subunit in *Drosophila* mutants before and after the acquisition of tolerance was studied. The experiments were aimed at finding out how the rewarding properties of ethanol influence its direct effect on G proteins expression.

These experiments have shown that chronic ethanol exposure significantly affected both the basal level and the acute response level of G protein mRNA but these changes were subunit specific and with potentially important difference between the strains. G_q subunit in the G_q mutants reduced significantly in the basal level and acute response in chronic flies (Figure 5.5). The expression of G_i and G_q subunits in the G_i mutants were significantly reduced in the acute response in chronic flies (Figure 5.7). G_i subunit in naïve flies increased significantly and both the basal level and acute response in chronic flies reduced significantly. The significance of the change in G_q and G_i expression will be discussed in later sections.

Using flies lacking the Dop1R2 to measure the expression of G_i, G_q and G_s before and after tolerance, also showed a significant reduction in the acute response in chronic flies for both G_i and G_q subunits. Significant results were also observed by the G_q subunit in the basal level of chronically treated flies (Figure 5.9). The finding that the same type of changes were observed in both the wild-type and Dop1R2 suggests that the observed

alcohol induced changes in G protein are not dependent on the D1-like dependent mechanism that have been associated with addiction (Le Foll *et al*., 2009).

5.3.3. Evaluation of the type of mutation with respect to the observed effect

The mutation in the G_q gene is a point mutation, which leads to reduced activity of G_q protein function (Kain *et al*., 2008). The fact that the mRNA for G_q gene was detected by qRT-PCR is not surprising because the mutation itself would not affect it's expression and translation to protein. This may lead to the possibility of the protein forming a complex with βγ subunits without being functional.

The strain 17672 was developed by inserting a P-element in the G_i gene (Bellen *et al*., 2004). It is thus rather surprising that in the strain 17672, mRNA could be detected at all. In fact, analysis of the location of the insertion indicates that the P element was inserted in the large intron 1 (Figure 5.2). It would thus seem that the insertion does not affect the splicing mechanism and that a 'normal' G_i mRNA is produced as the primers used in qRT-PCR spanned exons 1 and 2. In addition, it would have been useful to carry out experiments such as PCR with primers spanning all the exons of the G_i gene.

To ensure that the product of the qRT-PCR was indeed the expected sequence, the PCR product for G_i amplification was sequenced and confirmed to be as expected (Appendix 5.1). Expression of this mutated gene has not been reported elsewhere. In retrospect, this indicates that this was not a good choice of mutants for the intended analysis of a strain lacking G_i gene. However, as it will be discussed later it fortuitously acts as a very useful control for this study.

The Dop1R2 mutation has been produced via insertion of the *Minos* element and it has been reported to cause a small (15%) but significant reduction of mRNA expression (Liu *et al*., 2012). In the study of Liu *et al* (2012), this mutant has been used to

demonstrate that this mutation does not affect specific sleep behaviour, however, the fact that sufficient protein expression may still present in the mutant should be considered but it has not been addressed in the literature or in this study.

The most important finding in the present study is the demonstration that there was a reproducible reduction in the acute effect in chronic flies. Ethanol administration could be causing a rapid degradation of *Drosophila* mRNA considering the period of treatment is small (30 min of exposure ethanol). The lower level of G protein subunit such as G_q in *Drosophila* could be due to a high rate of turnover of the G_q pre mRNA in the nucleus. For example, RNA tends to fold into molecules with varying secondary and tertiary structures that can influence the accessibility of translation initiation codon to the ribosome.

In addition, the observed reduction may be as a result of alterations in microRNA (miRNA) levels. MicroRNAs are small noncoding RNA molecules that regulate mRNA and protein expression and are known to play significant roles in the actions of ethanol (Miranda *et al*., 2010). It was recently discovered that a miRNA-based mechanism could contribute to the development of molecular tolerance to ethanol (Pietrzykowski and Treistman, 2008; Miranda *et al*., 2010).

5.3.4. Ethanol-induced behaviour and gene expression differences among *Drosophila* mutants

Ethanol-induced behaviour demonstrated that flies acquire tolerance when exposed repeatedly to ethanol. In the mutant, G_q and strain 17672, they had lower ethanol sensitivity compared to Dop1R2 mutants after tolerance. This clearly suggests that at least for G_q, the functional protein is not essential for the development of tolerance or that other genes can substitute them in the process. It should be noted that the observed

changes in ethanol sensitivity in the mutant strains, may not be due to the mutation itself but simply because in the selection process of isolating the mutants with different sensitivity to alcohol were selected. Indeed, the experiments described in chapter 3 demonstrate that the subpopulations of *Drosophila* (early and late responders) can be isolated based on their response to ethanol.

It should be noted that there was no correlation between the behaviour of the fly strains and their gene expression as strain 17672 and Dop1R2 mutant displayed lower and higher sensitivity to alcohol respectively, but had the same changes in gene expression. In summary, in all three *Drosophila* strains (G_q, G_i and Dop1R2), the G_q subunit is significantly reduced in all the mutant populations following chronic treatment with ethanol while G_i subunit is reduced significantly in strain 17672 and Dop1R2 mutant but not in the G_q mutants (Figure 5.10) suggesting that the inactivation of G_q prevents the reduction of G_i. This would indicate a potential sequential response in terms of G protein gene expression where the reduction of G_q leads to a reduction of G_i.

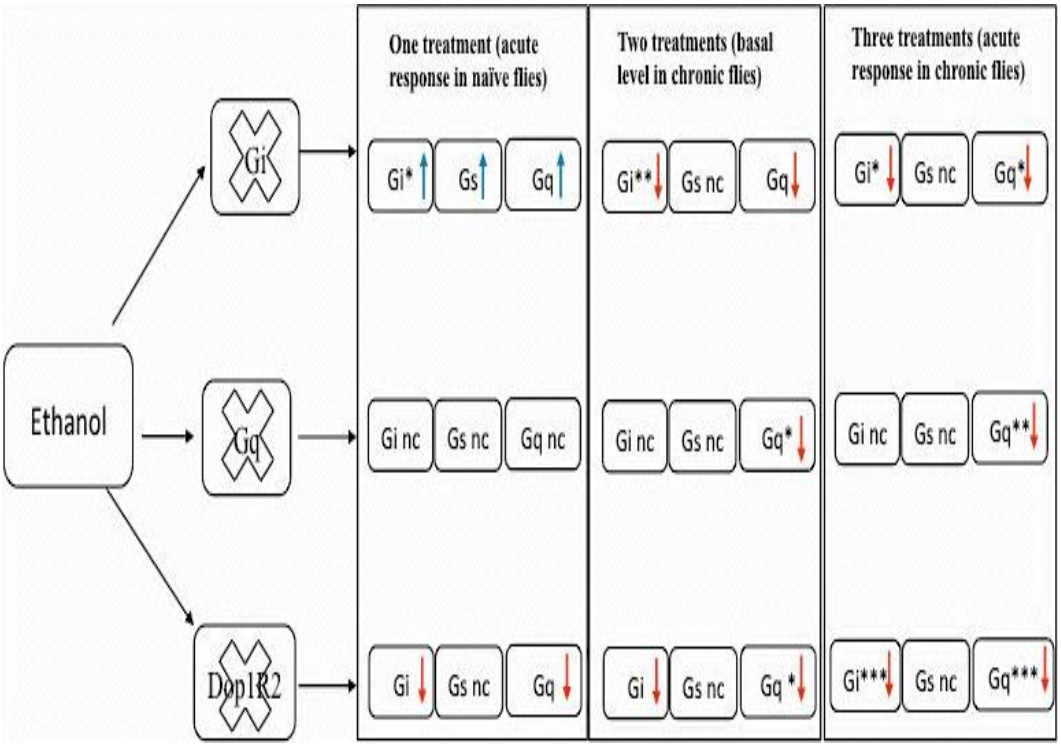

Figure 5.10. Summary of results obtained from ethanol-induced gene expression in *Drosophila* mutants as compared to naive flies

(One treatment: acute response, two treatments: basal expression in chronic flies, three treatments: acute expression in chronic flies). (*P<0.05, **P<0.01, ***P<0.001, nc: no change). Red arrow indicates decrease while blue arrow indicates increase.

5.3.5. How these changes might affect the signalling pathways

The effect of ethanol-induced gene expression on G_q and G_i subunits could suggest a possible role in their respective signalling pathways. The observed significant decrease in G_q expression (acute response in chronic flies) in all mutant strains may lead to the possibility of ethanol exposure causing a down regulation of the phospholipase C (PLC) / protein kinase C (PKC) dependent pathway. Reduced activity of PLC may predict decreased levels of free Gα subtypes coupled to PLC. The role of the PLC/ PKC dependent pathway has been less investigated with regards to the potential involvement

in ethanol administration. Although, the $G\alpha_{(q/11)}$-dependent PKC activity has been proposed to be involved in the development of sensitization with regard to abused drugs such as cocaine (Carrasco *et al*., 2003) and morphine (Narita *et al*., 2001).

The inhibitory G protein (G_i) is known to inhibit the cAMP/PKA dependent pathway (Neer, 1997). The significant decrease observed in G_i expression (acute response in chronic flies) in both G_i and Dop1R2 mutants, may suggest that seeking ethanol reward is accompanied by or occurs as a result of reduction of the cAMP pathway in various brain regions. Although, G_i subunit signal acts through a different pathway compared to Gq subunit, these two pathways may cooperate and in some situations, their interaction may be necessary for the induction of cellular plasticity (Kurose, 2003). The role of such an interaction in the central nervous system is not yet clear; nevertheless it might be important for neuronal plasticity.

Another possible model to describe how ethanol affects G protein expression could be due alterations in the expression of immediate early genes such as cyclic-AMP response binding protein (CREB) or *fos* protein (Kitanaka *et al*., 2008). CREB and *fos* protein have been implicated as underlying some of the long-lasting changes in neural gene expression following ethanol exposure (Nestler, 2000). Previous studies have demonstrated that ethanol treatments can induce changes in the levels of phosphorylated CREB in several brain regions (Li *et al*., 2003, Misra and Pandey, 2003, Yang *et al*., 2003).

The observed changes in G protein gene expression could have significant physiological functions. The prefrontal cortex has been demonstrated to be affected by changes in G protein gene expression (Kaewsuk *et al*., 2001, Zelek-Molik *et al*., 2012). Taking the mechanism of G protein into consideration, a reduction in one type of G protein subtype could lead to a switch of the normal association of the subtype of G protein associated with a particular receptor. If that's the case, a different response to ethanol will be

observed. In order to understand the significance of how ethanol administration alters G protein gene expression, it will be necessary to determine whether sustained changes could play a role in other drug-related behaviour such as withdrawal and relapse into drug use.

5.4. Conclusion

The main conclusions are:

- The different inbred lines of flies showed some significant changes in G protein expression, thereby confirming the trend observed in wild-type flies. The fact that the mutant strains are showing statistically significant changes in G protein gene expression is probably not due to the mutations themselves but rather to the fact that the mutant strains are more genetically homogenous than the wild-type population.
- The effect of the insertion-mutation needs to be treated with caution due to low effect on the mRNA expression. Even though the changes observed are statistically significant, they are small changes and may not have physiological relevance.
- An interesting result is the lack of change of G_i subunit in G_q mutants, as this could indicate a possible crosstalk between different signaling pathways, as discussed in Chapter 6.

Information on G protein gene expression could account for numerous drug-induced behaviours and could also be of importance in understanding human psychiatric disorders which share a substantial with addiction (Hudson *et al*., 1993; Lee *et al*., 2004).

Chapter 6: Discussion and future directions

6.1. Major findings

Alcohol addiction is a complex disease with important psychological and social causes and consequences, which occurs as a response to multiple administration of alcohol. Most changes in the brain associated with alcohol addiction happen gradually over time in response to chronic alcohol use. These gradually developing changes can persist for a long time after cessation of chronic alcohol exposure (Most *et al*., 2014). The adaptive changes that characterize an addictive state include tolerance, sensitization, dependence and withdrawal. The molecular changes that elicit these behavioural changes are thought to occur predominantly in the mesocorticolimbic dopaminergic system (comprising of the ventral tegmental area and nucleus accumbens), a reward centre of the brain (Chao and Nestler, 2004; Flatscher-Bader *et al*., 2006).

A wide array of studies has suggested that ethanol does not interact with a specific receptor, rather it alters the activities of several membrane receptors (NMDA and $GABA_A$), signalling elements and transcription factors. In addition, ethanol affects most other neurochemical and endocrine systems (Diamond and Gordon, 1997). Dopaminergeric and opioid systems are also affected by alcohol, mainly in regard to the brain reward system. Furthermore, neuronal second messenger pathways have been affected by alcohol. Regulation of gene expression of these second messengers provides an important platform for the understanding of ethanol administration. The literature on the effects of ethanol administration on G proteins is rather limited and pertains mostly to western blot analysis of G_s, G_i, G_o and Gβ proteins (Guillen *et al*., 2003; Hatta *et al*., 1994; Saito *et al*., 2002; Wenrich *et al*., 1998). Studies examining the mRNA expression of genes encoding G proteins are particularly scarce. With this gap in the literature in mind, this investigation has focused on the acute and chronic effects of

ethanol administration on mRNA expression of the Gα subunits within a model where *Drosophila* develops behavioural tolerance to ethanol.

Firstly, sensitivity and tolerance to the sedating effects of ethanol were studied in wild-type *Drosophila* (Chapter 3). *Drosophila* has been classed as a powerful model for studying the genes underlying acute ethanol responses (Devineni and Heberlein, 2010). As outlined in Chapter 3, a wide array of assays has been used in recent years to measure the effects of ethanol on *Drosophila*'s behaviour (Bainton *et al*., 2000; Bhandari *et al*., 2009; Devineni and Heberlein, 2009; Maples and Rothenfluh, 2011; Rothenfluh *et al*., 2006; Singh and Heberlein, 2000). Wild-type *Drosophila* exposed to ethanol over a period of three days, allowing 24h recovery period between each exposure, had higher ST50 (time to 50% sedation) than flies exposed once, thus demonstrating a manifestation of ethanol tolerance (Chapter 3, Figure 3.2). Due to the variations observed between individual flies, subpopulations (early and late responders) of wild-type *Drosophila* were generated based on how early or late they respond to ethanol (Chapter 3, Figure 3.3). These sets of flies (early and late responders), developed ethanol tolerance during recurrent ethanol exposure, indicating that they differed in terms of their intrinsic sensitivity to the effect of ethanol rather than in the tolerance response. First and second generations of the early and late responders maintained these behavioural characteristics demonstrating the role of genetic components in their behaviour.

The validation of ethanol tolerance demonstrated by both wild-type and subpopulations of *Drosophila* led to the investigation of G protein gene expression changes. mRNA was extracted from the head of female *Drosophila* (wild-type and subpopulations) that had been exposed to ethanol vapours either zero, one or three times for 30min to determine any changes in the acute response to ethanol. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure G protein mRNA

expression following chronic ethanol administration (Chapter 4) at three points namely: 1h after one treatment of ethanol (acute response in naïve flies), 24h after two treatments of ethanol (basal level in chronic flies), 1h after three treatments to ethanol (acute response in chronic flies). The changes of G proteins are expressed in comparison to the level of β-actin mRNA (Section 2.7 and Appendix 2.3), which does not appear to be affected by the ethanol treatment, as it does not show changes compared to other housekeeping proteins (Ponton *et al*., 2011). All Gα subunits namely G_i, G_o, G_f, G_q, G_s and cta proteins were initially measured in wild-type *Drosophila*. The initial findings confirmed that mRNA encoding all Gα subunits, were expressed in fly head and some ethanol-induced changes were observed but none reached a significance level predominantly due to variation in the response observed. However, measurements of G_i, G_q and G_s mRNA expression in subpopulations (early and late responders), showed less variable responses both in the basal levels and acute responses. In the early subpopulation, a trend (not statistically significant) in the reduction of G_i and G_q but not G_s was observed (Chapter 4, Figure 4.6).

In order to overcome some of the issues of variability among the wild-type population and subpopulations, and in order to try to begin to elucidate the mechanisms that may be responsible for the observed changes in G protein expression, specific G protein (G_i and G_q) and dopamine 1-like receptor 2 (Dop1R2) *Drosophila* strains were studied (Chapter 5). Initially, the ethanol-induced tolerance response of these strains was evaluated and some behavioural differences were observed but as discussed later, these may not necessarily be related to the mutation carried by the flies. Measurements of mRNA for G_i, G_q and G_s in these mutants at the above-described points of ethanol treatment (naïve-basal, acute, chronic-basal, chronic-acute) resulted in statistically significant changes, which are summarized in Figure 5.10 (Chapter 5).

One of the key outcomes of these studies is that, they demonstrate that indeed gene expression of some G proteins is affected by alcohol intake. Additionally, these studies suggest that the ethanol-induced changes in G_i expression may be dependent on the expression of G_q. If confirmed, this would expand our understanding of ethanol-induced molecular and behavioural changes.

In this final chapter, the validity and significance of the findings of ethanol-induced behaviours and gene expression will be analysed. This will be followed by a discussion on the strategies needed to further validate the results by methods such as western blots. To elucidate potential mechanisms for the observed changes, computational prediction of microRNA (miRNA) targets of the expressed G protein subunits will be presented. Finally, further possible directions for future work are considered.

6.2. Critical evaluation of findings

6.2.1. Ethanol-induced tolerance

In mammals, including humans, chronic alcohol use leads to the development of tolerance, simply defined as the acquired resistance to the sedating effects of ethanol (Wolf and Heberlein, 2003). Tolerance is one of the DSM-V criteria for alcohol related disorder (*Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-V) in 2013) and has been correlated with heavy drinking and alcohol abuse (Schuckit *et al*., 2009). Tolerance can be attained either by more efficient removal of alcohol from the body (metabolic or pharmacokinetic tolerance) or by adaptations in neural function (functional or pharmacodynamics tolerance) and reflect changes in neurophysiology (Berger *et al*., 2004; Wolf and Heberlein, 2003).

Drosophila have been previously described as an established model for rapid tolerance, a type of tolerance induced by a single intoxicating dose of ethanol and measured as a reduction in sensitivity observed upon administration of a second dose of ethanol delivered at a time when the initial exposure is completely metabolized (Berger *et al*., 2004). Two previous studies have examined rapid tolerance in *Drosophila*. One study found that flies that were exposed twice sequentially to ethanol vapour, displayed an increase in mean elution time (a measure of alcohol sensitivity) between the first and second exposure (Scholz *et al*., 2000). The other study, measured rapid tolerance after repeated ethanol injections in flies and obtained findings similar to those described by Scholz *et al*. (Dzitoyeva *et al*., 2003). This study has confirmed that in wild-type *Drosophila* and selected subpopulations (early and late responders) and mutant lines, tolerance can be induced by multiple exposures and can be measured in terms of ST50.

It is important to compare the approach used in measuring tolerance with previous studies in *Drosophila*. Most studies examining the effects of multiple ethanol exposures have focused on the sedative effects of ethanol. However, flies that had previously been exposed to ethanol showed an earlier onset and overall increase in ethanol hyperactivity (Kong *et al*., 2010). Ethanol tolerance appears to be robust to variations in the tolerance protocol, as different studies (Berger *et al*., 2004, Cowmeadow *et al*., 2005, Dzitoyeva *et al*., 2003, Scholz *et al*., 2000) vary significantly in the timing and concentration of ethanol exposure.

The main finding is that tolerance was observed in all *Drosophila* population studied in this thesis: wild-type, early responders and the three different mutants. Thus tolerance is an intrinsic response in *Drosophila* and is not dependent on alcohol sensitivity, signalling pathways initiated by G_q or by small reduction of Dop1R2 expression.

6.2.2. Variation in sensitivity to ethanol

Using the ethanol sensitivity assay, variations were observed within the population of wild-type *Drosophila*, which led to the isolation of early and late responders to ethanol. To the author's knowledge, this is the first study describing the selection of subpopulations of *Drosophila* with different responses to ethanol within the wild-type population as opposed to the isolation of behavioural variants induced by either chemical modification of DNA (Kain *et al*., 2008) or transposon mutagenesis (Bellen *et al*., 2004; Metaxakis *et al*., 2005). This finding correlates well with the evidence of differences in the response to alcohol and risk of addiction in humans.

These differences in susceptibility to alcohol have been linked to genetic variation (polymorphisms) in rodents (Crabbe, 2014). The hypothesis that genetic factors play a role in alcohol dependence have been further supported by a variety of observations; first, the risk of developing alcohol dependence is higher in relatives of alcoholics; second, among the offspring of alcohol-dependent subjects, identical twins have a higher correlation of alcoholism than fraternal twins or full siblings and thirdly, the adopted children of alcoholics have the same increased risk for alcoholism as offspring raised by their alcohol dependent parents (Cotton, 1979; Goodwin *et al*., 1974; Prescott and Kandler, 1999). In addition, a small number of genes known as endophenotypes (intermediate phenotypes that influence susceptibility to alcohol dependence) might directly influence alcohol dependence (Hines *et al*., 2005, Mayfield *et al*., 2008). Also, variations in receptor genes such as $GABA_A$ and cholinergic receptors, carries an associated predisposition towards alcohol dependence in relation to alcohol-induced behaviour (Mayfield *et al*., 2008).

Significant variation in the degree of behavioural response to ethanol has also been observed in rats and humans and does not reflect differences in ethanol metabolism (Begleiter and Porjesz, 1977). Studies have reported that alcohol-preferring rats are

innately less sensitive to the effects of alcohol compared to non-alcohol preferring rats (Bell *et al*., 2006). This also correlates with the observation described in this thesis, that late responders are less sensitive to ethanol. Individuals with a lower level of response to ethanol have a greater likelihood of becoming alcoholics (Rodriguez *et al*., 1993; Schuckit and Smith, 1996), probably because they can consume more alcohol.

6.2.3. Variation in ethanol-induced G protein mRNA expression

The observed behavioural variation within the wild-type *Drosophila* population, complicated the measurements of ethanol-induced G protein mRNA expression and probably was a major cause for variations in the data obtained from G protein expression using qRT-PCR. The impact that this variation in the sensitivity to ethanol had on this project can be summarized as follows:

1) The variation in the level of mRNA expression in replicate samples with the same treatment has been an ongoing problem of this project.

2) In order to minimize variation, it was necessary to optimize the technique in terms of carefully rearing the flies (amount of food, incubator temperature (25^{o}C), incubator light cycle, age of the flies, and length of CO_2 exposure).

3) This variation has been reduced by selecting subpopulations of flies (early and late responders).

4) This variation has been further reduced by using mutant flies which are genetically more homogenous. Although, using mutant flies provided more homogenous results, it may skew the results because of the mutation. However, in this study, different unrelated mutants used showed similar ethanol-induced behaviour and mRNA response.

6.2.4. Behavioural differences in mutants

The mutant population of *Drosophila* exhibited increased (Dop1R2) and decreased (G_i and G_q) sensitivity to ethanol. They also displayed the ability to develop tolerance to the sedating effects of ethanol, thereby confirming that mutations or insertions in specific G proteins or Dop1R2 genes did not block the induction and maintenance of tolerance. During the course of this study, it was identified that the G_i mutants (which carry a P-element insertion in the first intron of the G_i gene) probably produces a normal transcript after splicing. Additionally, the Dop1R2 mutants have a relatively small reduction of mRNA expression (Liu *et al*., 2012). Therefore, it is not possible to conclude whether the mutations/insertions can be linked to the observed behavioural changes (differences in sensitivity to ethanol) nor conclude that because the genes are putatively mutated, they are not involved in tolerance, which was maintained. It is also possible that the effect on these genes is compensated by genes producing similar proteins.

The behavioural differences (in terms of sensitivity to ethanol) may in fact be related to factors other than the specific mutation. The study of early and late responders indicated that there are genetic background differences within the *Drosophila* population and the different mutants could have different genetic background as well.

6.2.5. Gene expression in *Drosophila* mutants

The experiments in this thesis were aimed at finding out whether the rewarding properties of ethanol affect G protein gene expression in *Drosophila*. Changes of some G protein subunits were indeed observed in a number of different ethanol treatments and population of flies. This discussion will start from analysing the results that were statistically significant and then compare them to experiments where similar trends were

observed. The development of ethanol-induced tolerance was associated with a significant decrease in the levels of G_i and G_q mRNAs in the head of strain 17672 and Dop1R2 mutant after chronic ethanol treatment. The G_q mutants also showed ethanol-induced decrease in G_q but not in G_i expression (Table 6.1).

It is important to note that the observed changes in gene expression were specific to G_i and G_q and other subunits such as G_s did not change. Also, it is interesting to note that for both G_i and G_q, a decrease in the expression of both the basal level of chronic flies and further decrease in the acute response in chronically treated flies were observed. As discussed later, in order to explain a decrease in the acute response, one would have to hypothesize either the interruption of replenishment of a rapidly turning-over pool of mRNA and/or active mRNA degradation by processes such as microRNAs (miRNAs).

Furthermore, the fact that the non-statistically significant changes observed in the early responders follow the same trend than the statistically significant changes observed in the mutant flies, suggest that the observation in the wild-type flies and early responders actually reflect a biological phenomenon. It would be useful to develop a mutant that behaves more like the late responders, which do not show these mRNA changes.

6.2.6. Gene expression differences in *Drosophila* strains

In Chapter 4, wild-type and subpopulations of *Drosophila* (early and late responders to ethanol) were used to test ethanol-induced gene expression. While in Chapter 5, G protein changes were measured in three fly strains.

Similar effects (but not at a statistically significant level) were observed in both subunits between the wild-type, early responders and G_q mutant and strain 17672 (Table 6.1). It can be hypothesized that one of the reasons that changes were observed at a significant level in the mutants and at lower significance in the wild-type is because the mutant

strains are more genetically homogenous than the wild-type population and thus there is less behavioural and gene expression variation within the population.

This findings suggests that within a more homogenous population of *Drosophila*, ethanol induced G protein gene expression changes can be detected but the persistence of this effect in flies with a reduced D1-like expression suggests that the changes in expression of G_q and G_i are not dependent on full level of expression the D1 receptor mechanisms associated with addictive drugs.

Table 6.1. Summary of results of the changes in the G protein subunits in all used *Drosophila* strains

Strain	Acute response in naive flies	Basal level in chronic flies	Acute response in chronic flies
Wild-type	Gi ↑, Gq ↓	Gi Gq ↓ ↓	NC Gi , Gq ↓
Early responders to ethanol	NC	Gi Gq ↓ ↓	Gi ↑, Gq ↓
Late responders to ethanol	NC	NC Gi, Gq ↑	NC Gi, Gq ↓
Gq mutants	Gi ↑, Gq ↑	Gi NC, Gq ↓*	Gi NC, Gq **↓
Strain 17672	Gi ↑*, Gq ↑	Gi ↓**, Gq ↓	Gi ↓**, Gq ↓*
Dop1R2 mutants	Gi ↓, Gq ↓	Gi ↓, Gq ↓ *	Gi ↓***, Gq ↓ ***

(One treatment: acute response, two treatments: basal expression in chronic flies, three treatments: acute expression in chronic flies). (*P<0.05, **P<0.01, ***P<0.001, nc: no change).

6.2.7. Implications of findings on G protein activation

Many cellular signalling transduction pathways are associated with the stimulation of GPCR and subsequent activation of heterotrimeric G proteins (Oldham and Hamm, 2008; Wettschureck and Offermanns 2000). Activation of GPCR catalyses the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the G protein α subunit (Gα) leading to the trimeric complex dissociating and to the release of free Gα-GTP and Gβγ subunits, each of which may then interact with effector molecules (Boto *et al*., 2010). The G_i-GTP negatively affects adenylyl cyclase (AC) and G_q stimulates phospholipase C (PLC) isoforms (Clapham and Neer, 1993; Offermanns, 2003).

AC goes on to generate cyclic cAMP, which is required to activate cAMP-dependent protein kinase A (PKA) (Newton and Messing, 2006). In contrast, G_q activates beta-isoforms of phospholipase C (PLC), thereby leading to the production of inositol triphosphate 3 (IP3) and diacyglycerol (DAG). IP3 then releases intracellular calcium stores to increase intracellular calcium levels, while DAG activates protein kinase C (PKC). The changes observed in both G proteins mRNA expression in this thesis may be secondary effects downstream of ethanol-induced alterations of other receptors such as dopamine or acetylcholine receptors.

The specific activation of G_i and G_q signal transduction pathways by repeated ethanol treatment raises the question if the G protein subunits (G_i and G_q) work in conjunction in downstream pathways. Previous studies have shown that ethanol alters several cellular functions through modifications in G protein associated signalling. For instance, it was reported that pharmacological ethanol concentrations (10 – 100mM) affected G_q-mediated signalling pathway, causing changes in its downstream effector, PKC activity (Gonzales *et al*., 1986; Hoffman and Tabakoff, 1990).

The effect of ethanol on G_i-mediated signalling pathway has been less reported. For instance, addition of G_i to reconstituted systems has produced little or no inhibition of AC and in pertussis-toxin-treated membranes, the addition of βγ subunits of G proteins produced more inhibition than the G_i subunit (Smigel *et al*., 1984). These data supported the hypothesis that inhibition of AC occurs because activation and dissociation of G_i results in the release of βγ subunits that can interact with G_s (Hoffman and Tabakoff, 1990). This leads to changes in protein kinase A (PKA) activities. These findings are relevant because G proteins control diverse functions, including cell division, differentiation and in the case of the central nervous system (CNS), excitability regulation, synaptic transmission and disease (Wettschureck and Offermanns, 2005; Yevenes *et al*., 2011). Thus, it seems possible that ethanol might affect a number of cellular functions by altering G proteins.

6.2.8. Implications of findings on the signalling pathways

Ethanol has been reported to affect the activities of some signalling pathways, including cAMP-PKA dependent pathway (Wand *et al*., 2001; McBride et al., 2014) and PLC-PKC dependent pathway (Coe *et al*., 1996). In addition, extracellular signal-regulated kinases (ERK) have been reported to stimulate MAPK (mitogen-activated protein kinase) activation, including both receptors that couple to G_q and to G_i (Hawes *et al*., 1995, Lee and Messing, 2008, Newton and Messing, 2006). Although, G_i signals through a different intracellular pathway (PKA) than G_q (PKC), these two pathways may cooperate, and in some situations their interaction is necessary for the induction of cellular plasticity. The role of such interaction in the CNS is not yet clear, nevertheless it might be important for neuronal plasticity.

Several studies have implicated PKA in the intoxicating effects of ethanol (reviewed in Ron and Messing, 2013). Inhibition of PKA activity has shown decreased sensitivity to the hypnotic effects of ethanol (Thiele *et al*., 2000). In addition, PKA signalling has been reported to also regulate ethanol consumption. Mice deficient in RIIβ (gene of the PKA regulatory subunit), show reduced cAMP-stimulated PKA activity and increased ethanol intake (Thiele *et al*., 2000). However, other investigators have revealed that inhibition of PKA activity signalling increases acute effects of ethanol and decreases ethanol intake (Thorsell *et al*., 2007, Wand *et al*., 2001).

On the other hand, ethanol has been reported to activate, inhibit or have no effect on PKC signalling activity in vitro, depending on the experimental conditions (reviewed in Stubbs and Slater, 1999). One study suggests that ethanol was found to bind PKC isoforms and inhibit its activity when assayed in vitro (Das *et al*., 2009), while another study suggests that ethanol exposure activated PKC isoforms in intact cell systems (Jiang and Ye, 2003, Qi *et al*., 2007). Ethanol regulation of PKC isoforms is most likely to be indirect, due to modulation of upstream signalling pathways that generate DAG or that lead to phosphorylation of sites necessary for full kinase activity, such as the C-terminal hydrophobic motifs of PKC (Newton and Messing, 2006). In addition, ethanol has been reported to cause translocation of PKC from the Golgi to the perinucleus and PKC from the perinucleus to the cytoplasm (Gordon *et al*., 1997). Acute ethanol administration alters the distribution, whereas chronic administration increases the abundance and translocation of PKC in neural cells lines (Gordon *et al*., 1997).

Several lines of evidence have reported possible crosstalk between PKA and PKC cascades in cellular systems. Crosstalk between PKA and PKC signalling pathways play an important role in the activation of intracellular and intranuclear signal transduction cascades (Yao *et al*., 2008) and is necessary to regulate gene expression (Sengupta *et al*., 2007). Such crosstalk is important in the ventral tegmental area (VTA), where drugs

of abuse increase extracellular levels of dopamine and thereby activate dopamine 2 autoreceptors on dopaminergic neurons (Ron and Messing, 2013). This event leads to the activation of PKC and PKA, which phosphorylate and up regulate tyrosine hydroxylase (TH) and increase the production of dopamine (Yao *et al*., 2010). However, the molecular events underlying PKA/PKC crosstalk are not fully understood. Previous evidence has implicated PKC in the activation of AC and increasing the level of cAMP in intact cells and cell-free systems (Cooper *et al*., 1995). PKC have also been reported that PKA plays a role in the activation and translocation of PKC from the golgi to the perinucleus (Yao *et al*., 2008).

In this study, it was demonstrated that repeated ethanol administration reduces the mRNA expression of G_i subunit (associated with PKA) and G_q subunit (associated with PKC) in two fly strains that were tested except for the G_q mutants which suggest that indeed there could be cross-talk between PKC and PKA in the response to ethanol. It should be stressed that according to the literature while there may be doubts about the functional implications of the insertions in the G_i and Dop1R2 genes, there is clear evidence that the point mutation in the G_q mutant does produce a non-functional protein. Therefore it is possible to propose that the lack of changes in G_i expression in the G_q mutants is due to lack of a functional G_q. This would suggest that under normal circumstances in flies chronically treated with ethanol, G_q expression is reduced (Figure 6.1) which presumably leads to a reduction of PKC activity which could in turn reduce expression of G_i.

Figure 6.1 (A, B & C) presents a potential model of the general mechanisms affected by ethanol in both G_i and G_q. The studies here presented demonstrated a change in G_i and G_q, which would potentially affect PKA and PKC pathways respectively. The model suggests that the Gq/PKC pathways would influence the Gi/PKA pathway through changes of G_q expression. It would be interesting to try to demonstrate such a

mechanism by directly inhibiting PKC or artificially activating PKC in the G_q mutants. The reinforcing effect of the two major pathways inhibiting each other could be an important mechanism of addiction.

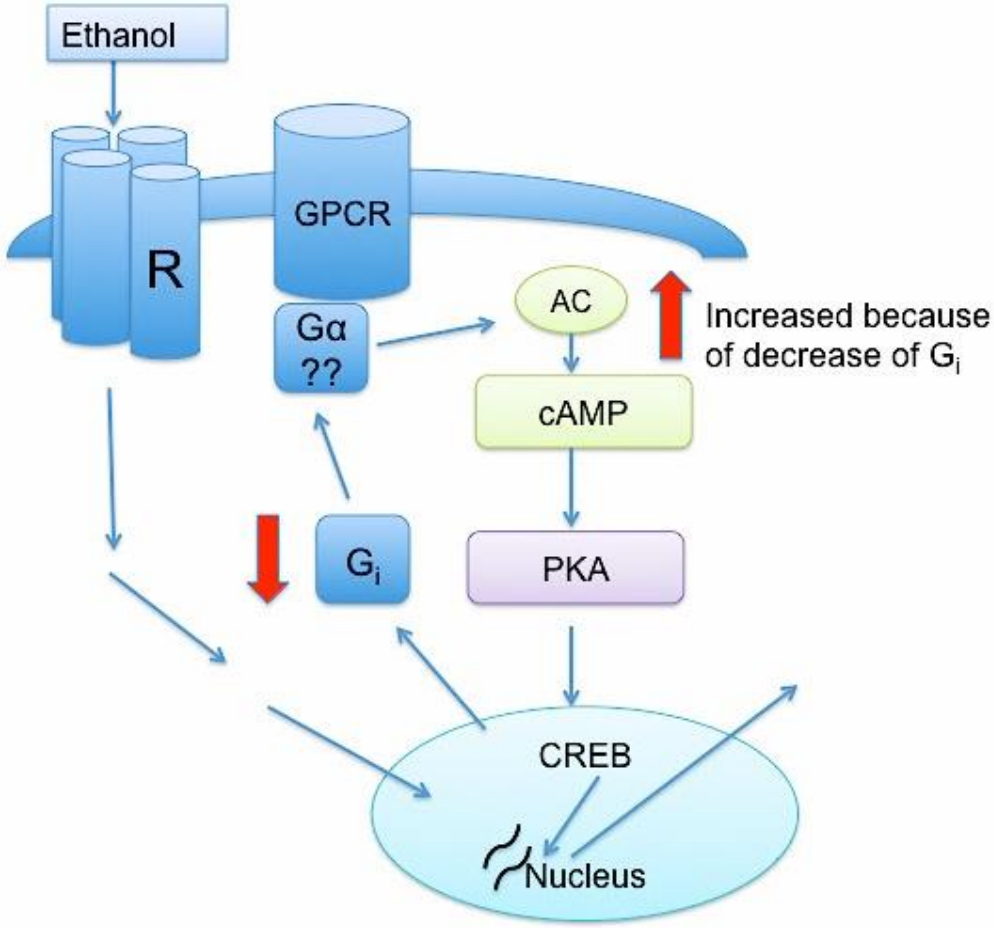

Figure 6.1. Presents potential models of the general mechanism affected by ethanol starting from its effect on receptors (R) leading to changes in gene expression.

(A) The model suggests that ethanol causes a change in G_i subunit expression, which could affect which G protein subtype is associated with GPCR. A reduction in G_i which normally inhibits Adenylyl Cyclase (AC) would result in an increase in AC activity and thus and increase in protein kinase A (PKA) activity and the phosphorylation of transcription factors such as cAMP-responsive binding protein (CREB) which would lead to further changes in gene expression.

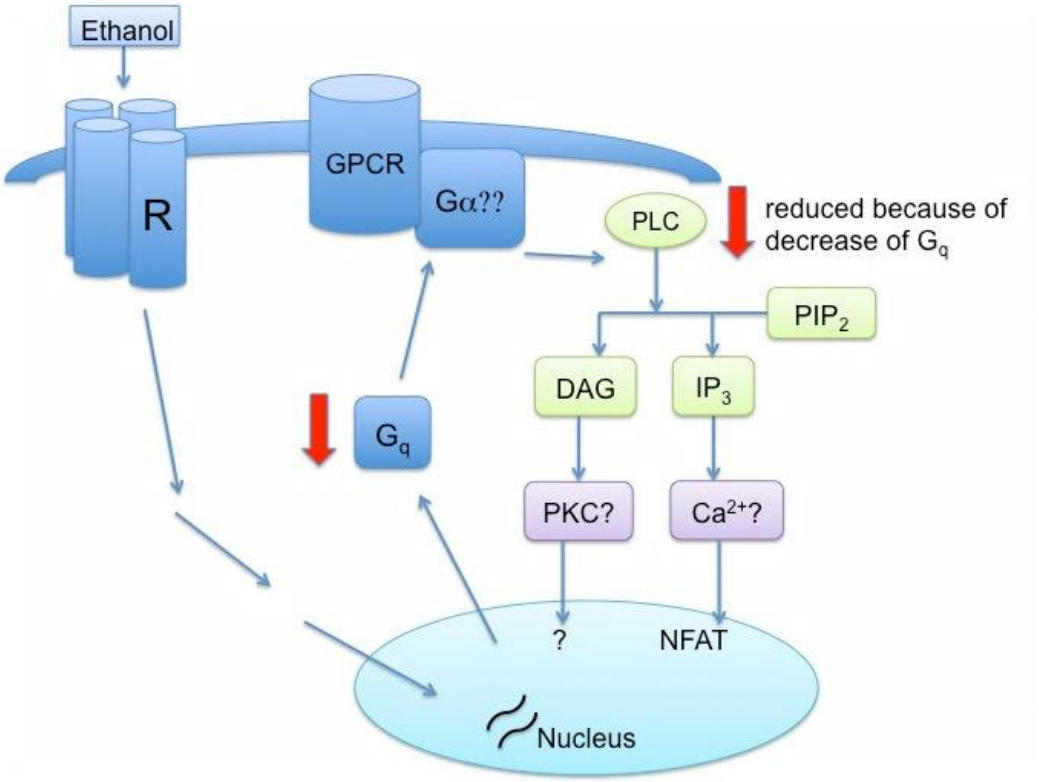

Figure 6.1(B) The model suggests that ethanol causes a change in G_q subunit expression, which could potentially affect which G protein subunit is associated with the G-protein coupled receptors (GPCR). Inhibition of G_q would decrease the activity of Phospholipase C (PLC) and consequently a reduction in Protein Kinase C (PKC) pathway and the phosphorylation of transcription factors such as nuclear factor of activated T-cells (NFAT) through Ca^{2+}, which would lead to changes in gene expression.

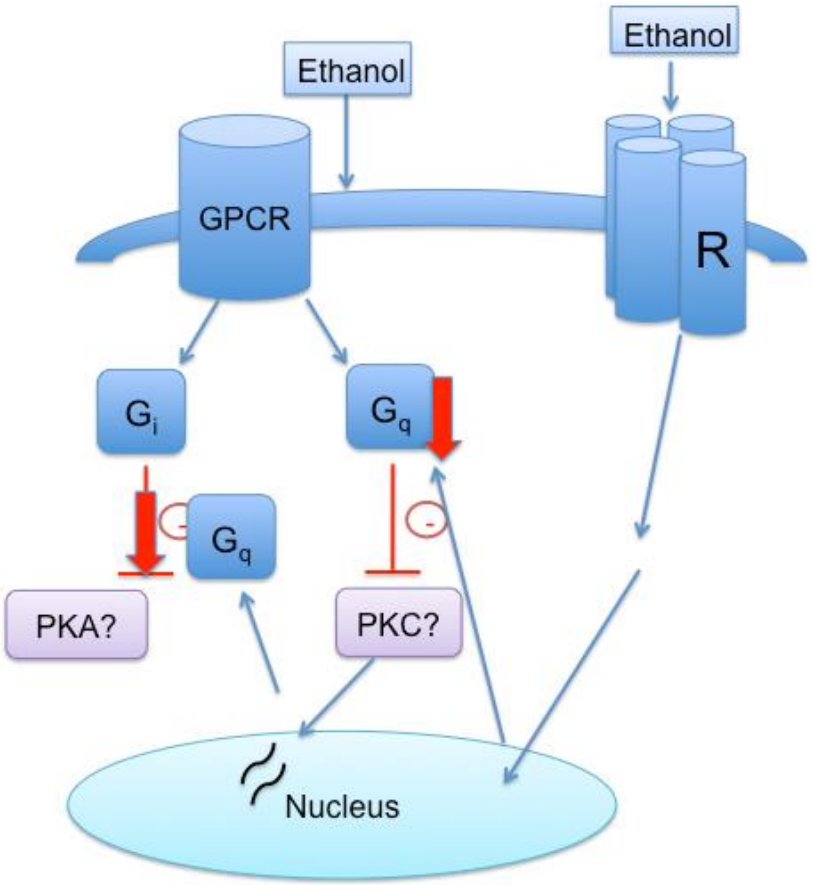

Figure 6.1(C) This model is based on the observation that in the fly strain 17672, where G_q in non-functional, the G_i decreases does no take place leading to the speculation that the G_i decrease in expression is dependent on G_q decrease.

6.2.9. Validation of G protein gene expression changes using western blot

In this study, western blot analysis of G proteins (G_i and G_q) in fly brain samples was attempted to confirm the observed mRNA gene expression of G_i and G_q (Section 4.2.4). Changes in mRNA levels often correlate with changes in protein level but there are also cases where this correlation does not hold and it is thus preferable to demonstrate directly changes in protein level (Schwanhausser *et al*., 2011).

There are no commercial antibodies proven to recognise *Drosophila* G proteins. Antibodies were selected on the basis of similarity of the antigenic peptides used for raising the antibodies and *Drosophila* protein sequence. Substantial efforts were carried

out to measure the protein level of G protein subunits, as this would further validate the significance of the changes in mRNA expression observed. Unfortunately, the only antibodies that showed some initial positive results (Chapter 4, Section 4.2.4) proved to be unstable and are no longer available.

The lack of functionality of the G protein antibodies have also been reported by other investigators (http://www.ihcworld.com/smf/index.php?topic=1986.0). It was thus not possible to continue this line of work.

6.3. Future directions

This thesis provides new evidence regarding ethanol-induced gene expression in *Drosophila*, however details of the mechanism of changes still need to be elucidated and as suggested, and the role of signalling pathways needs further explorations. It will also be interesting to identify whether changes in ethanol-induced gene expression of G proteins are associated with other addiction related behaviours such as withdrawal and relapse.

Investigation will also be needed to characterize the expression of these G protein subunits at a cellular level using immunocytochemistry. This could lead to the localization of the specific neurons in which the observed changes of G protein gene expression occur.

Additionally, it will also be interesting to investigate the potential role of the identified miRNA in the regulation of G protein expression using *Drosophila* Schneider 2 (S2) cell line (which are derived from *Drosophila* embryos) as described in Appendix 6.1. If a positive result is obtained for the miRNAs, it would be of interest to develop a mutant fly with inducible expression of a specific miRNA in the neurons identified in the immunocytochemistry study. In such mutants it may be possible to induce effect similar

to those induced by alcohol, which will open the possibility of using miRNA to intervene in addictive behaviour.

6.4. Significance of the findings and relevance to alcoholism

The results described in this study provide new insight into some of the cellular and molecular events mediated by ethanol. This study also provides a model for the investigation of the neuroadaptive processes that occur in response to acute and chronic ethanol exposure in *Drosophila melanogaster*. Ethanol's effects on the central nervous system are diverse and include changes in localization of the nucleus and post-translational modifications, gene expression and neuronal excitability (Most *et al*., 2014; Ron and Messing, 2013). It was shown here that repeated ethanol administration leads to reduction of mRNA expression of G_i and G_q subunit in an apparently interdependent manner, thereby potentially affecting PKA and PKC signalling pathways respectively via a possible crosstalk between these two signalling cascades. Moreover, ethanol activated PKA and PKC seems to play a role in drinking behaviours; mice lacking PKC show reduced operant ethanol self-administration (Olive *et al*., 2000) and inhibition of the cAMP/PKA signalling pathway generally increases sensitivity to ethanol sedation and reduces ethanol preference and consumption (Moore *et al*., 1998; Wand *et al*., 2001). Taken together with the results in this study, it is possible that drugs, which interfere with PKA and PKC crosstalk, might be potential therapeutics for alcoholism.

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Appendix

Appendix 2.1

The G protein variants for each gene are listed in the table below.

G protein subunits	Transcripts
G_i	1
G_q	7
G_o	10
G_s	5
G_f	1
cta	2
$G\beta_{76C}$	1
$G\beta_{13F}$	6
$G\beta_5$	1
$G\gamma_1$	3
$G\gamma_{30A}$	8

Alignment of G protein transcript variants using cta gene as an example. The Figure below shows the forward primer (shown in green) and reverse primer (shown in red) of cta template.

Figure 1. Alignment of Gα gene, cta transcript variants.

Cta have two transcript variants. The DNA sequences for both variants were obtained from NCBI and aligned using CLUSTALW2. A represents variant A, while B represents variant B.

Appendix 2.2

A melting curve analysis was carried out to ensure the product of primers were the expected size and not the result of primer dimers. The image below shows an example of a melting curve.

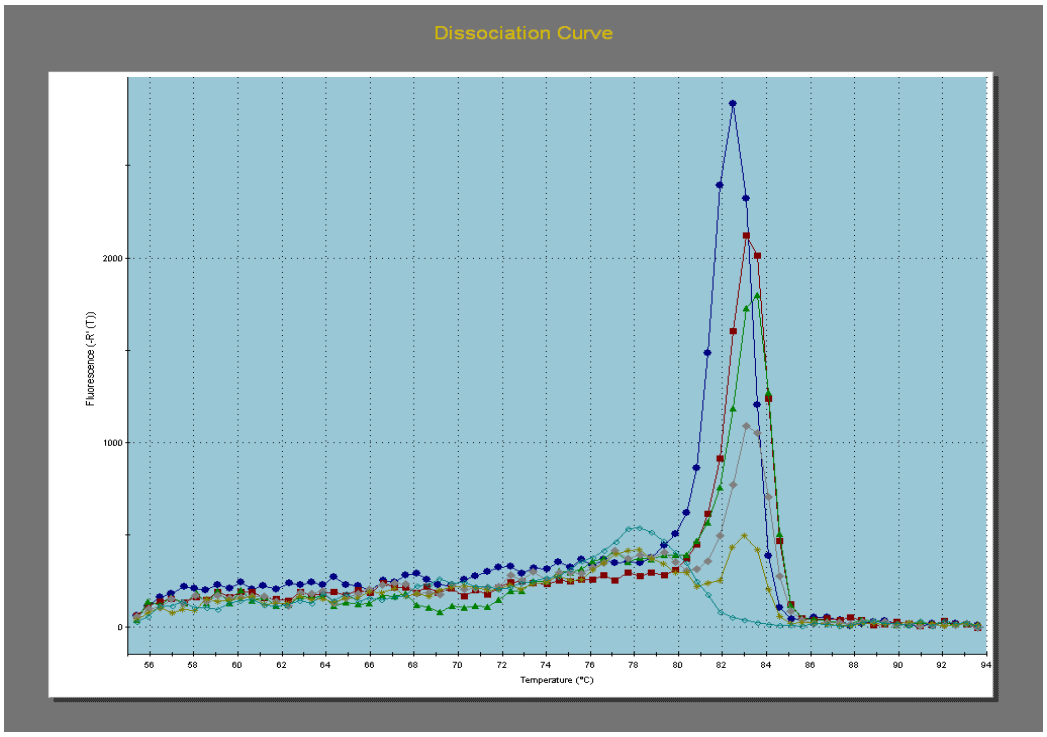


Figure 1. Image of a melting curve.

Change in fluorescence (y axis) with increasing temperature (x axis) is measured. As the temperature increases, the two strands of the amplicon separates to form single stranded DNA, causing the fluorescent intercalating dye to dissociate from the DNA and stop fluorescing.

Appendix 2.3

The table below shows a worked example of the calculation of relative expression using the comparative method ($2^{-\Delta\Delta Ct}$).

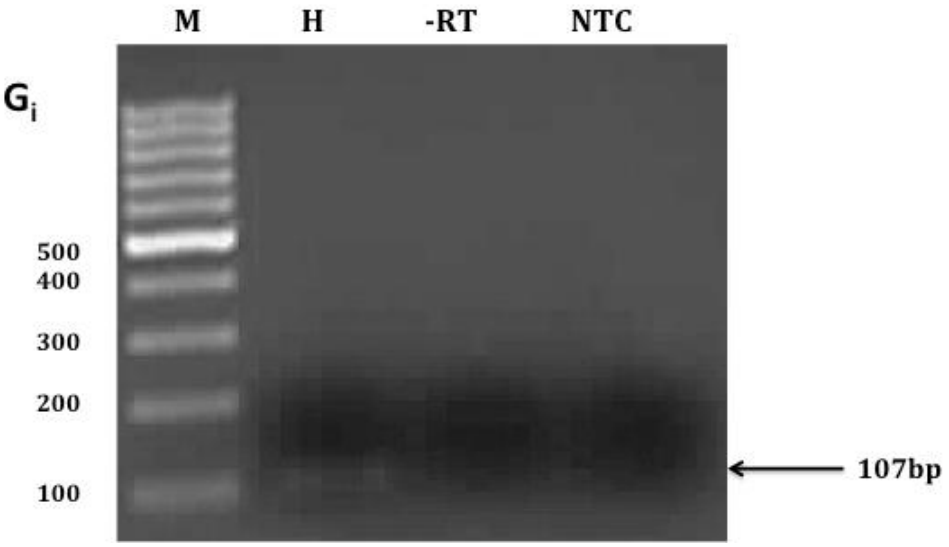
$2^{-\Delta\Delta Ct}$ method

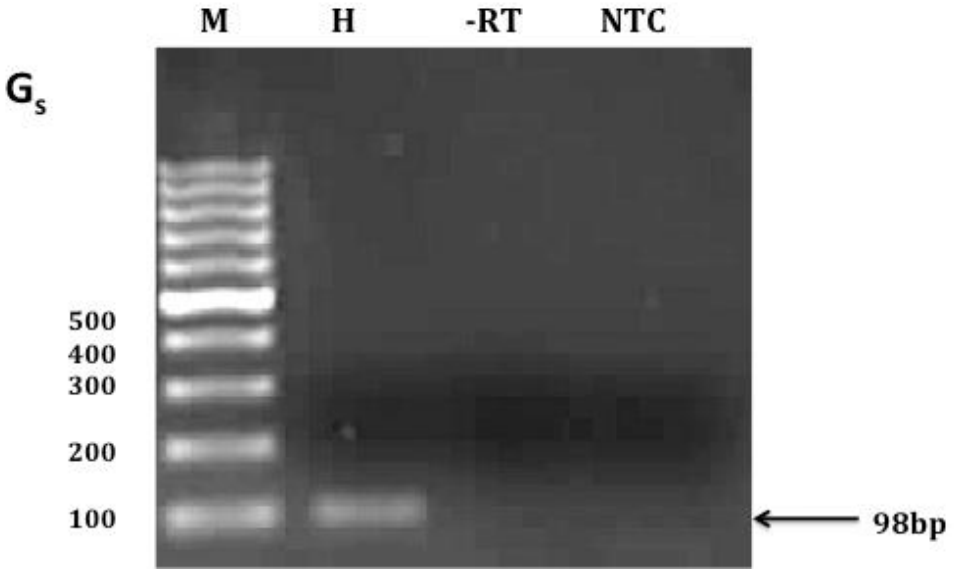
Sample	**Gq average Ct**	**Actin average Ct**
Control	28.23	26.67
One exposure	28.44	26.96
Before three exposures	28.24	26.08
Three exposures	25.77	25.95
Control: 2^(actin - Gq)	0.34	
One exposure: 2^(actin - Gq)	0.36	
Before three exposures: 2^(actin - Gq)	0.22	
Three exposures: 2^(actin - Gq)	1.13	
Ratio of expression - control	1	
Ratio of expression - One exposure	1.06	
Ratio of expression - Before three exposure	0.66	
Ratio of expression - Three exposure	3.34	

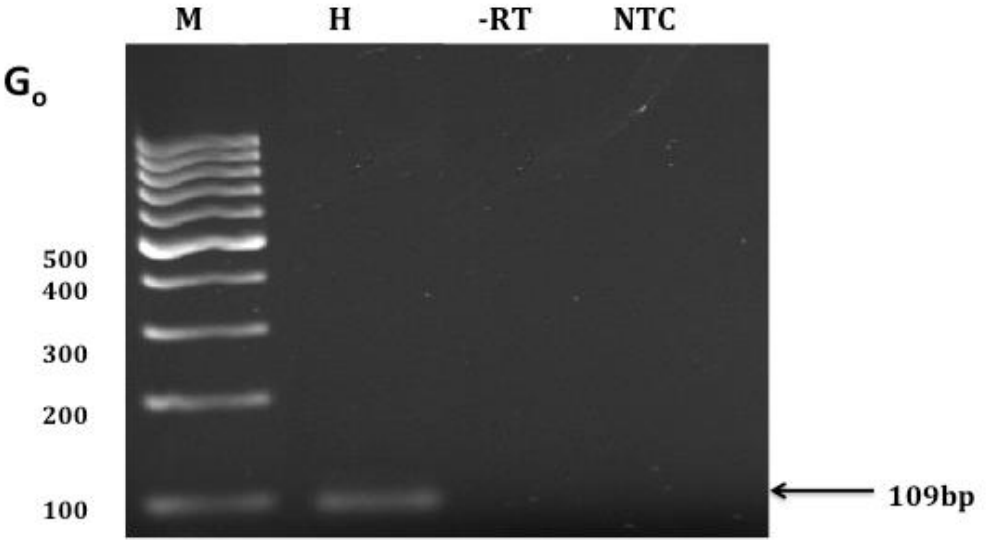
Appendix 4.1

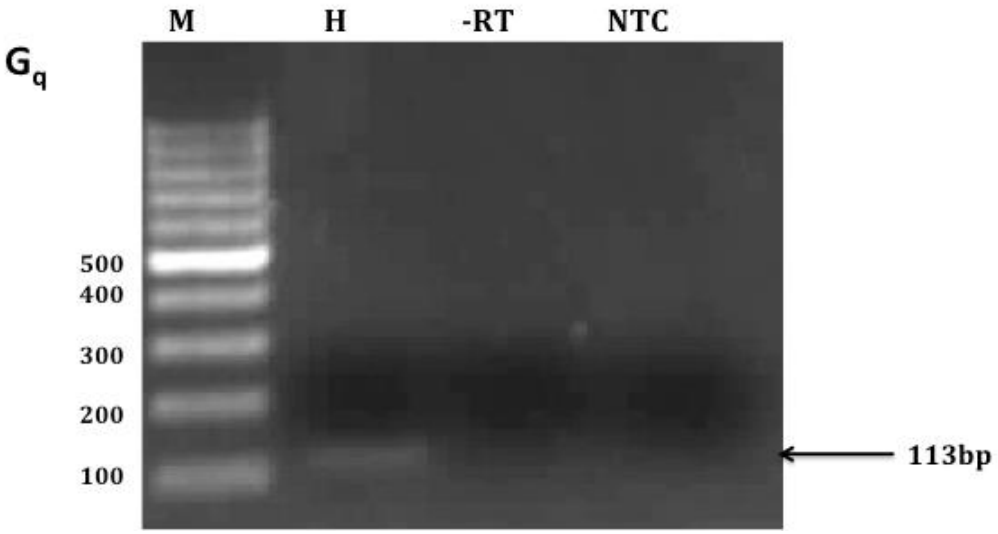
Full agarose gel images of G protein subunits below.

A) Expression of Gα genes (G_i, G_s, G_o, G_q, G_f and cta)






M
H
-RT
NTC
G_o
500
400
300
200
100
109bp


M
H
-RT
NTC
G_q
500
400
300
200
100
113bp

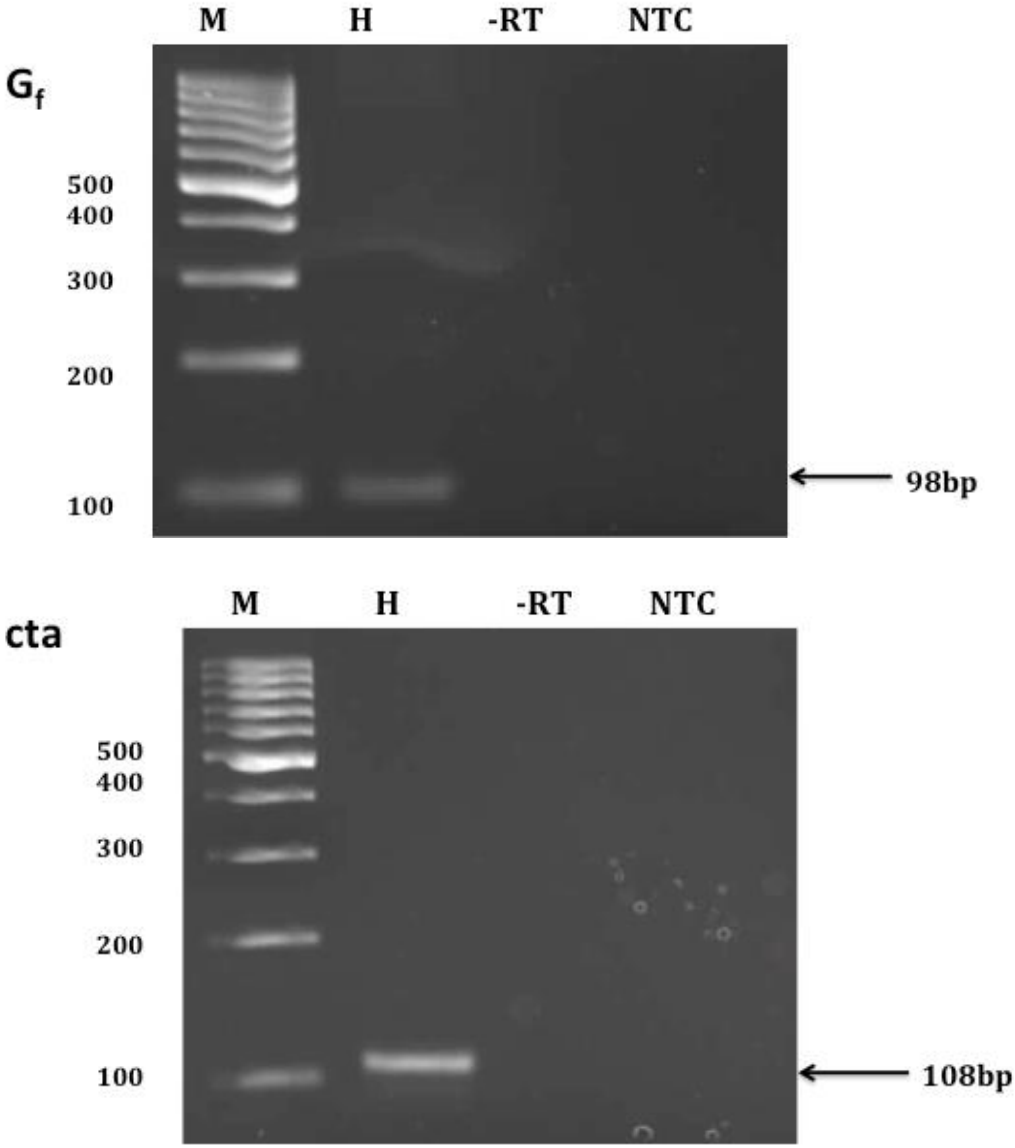
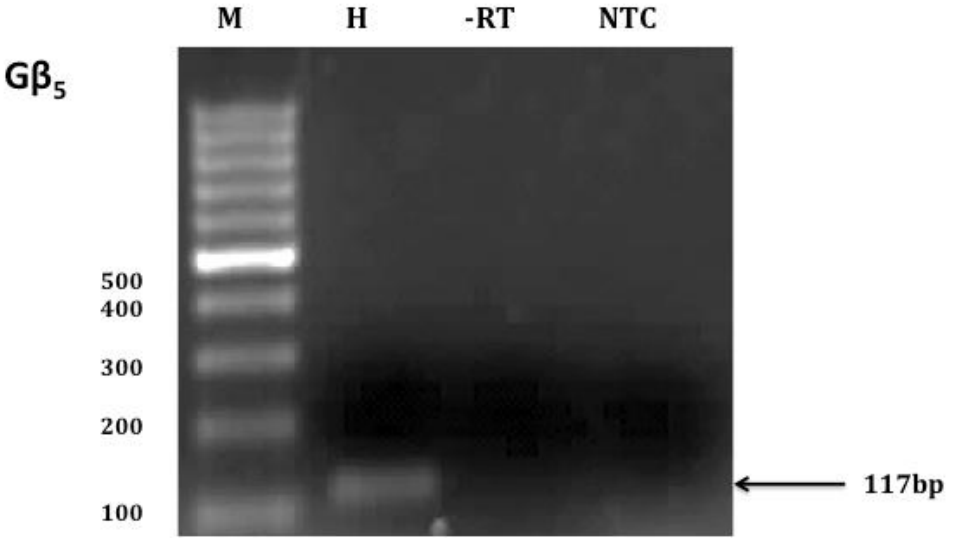
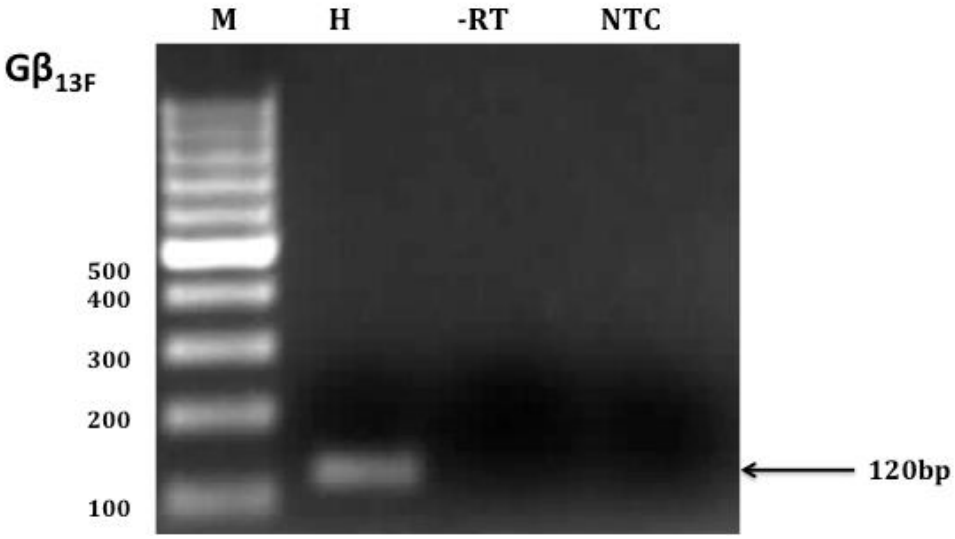


Figure 1. Expression analysis of Gα genes (G_i, G_s, G_o, G_q, G_f and cta) in the head of adult *Drosophila* using reverse transcriptase chain reaction (RT-PCR)

Target genes are denoted on the left of the figure and the amplification product size on the right, expressed in base pairs (bp). RNA was extracted from the head of the flies and this is identified at the top of each figure as follows: M: 100bp ladder; H: head; -RT (minus reverse transcriptase); NTC (No template control).

B) Expression of Gβ genes ($G\beta_5$, $G\beta_{13F}$ and $G\beta_{76C}$)





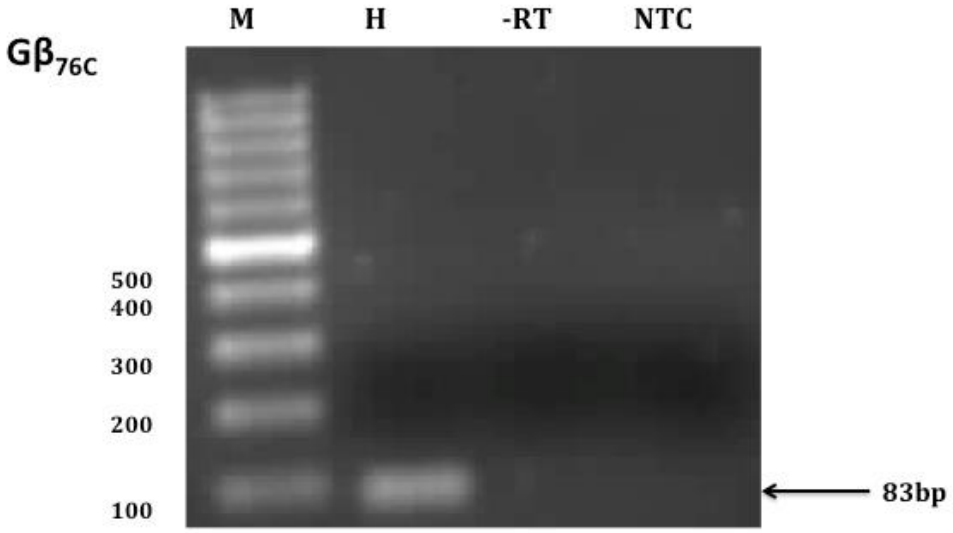


Figure 2. Expression analysis of Gβ genes (Gβ$_5$, Gβ$_{13F}$ and Gβ$_{76C}$) in the head of adult *Drosophila* using reverse transcriptase chain reaction (RT-PCR)

Target genes are denoted on the left of the figure and the amplification product size on the right, expressed in base pairs (bp). RNA was extracted from the head of the flies and this is identified at the top of each figure as follows: M: 100bp ladder; H: head; -RT (minus reverse transcriptase); NTC (No template control).

C) Expression of Gγ genes (Gγ$_1$ and Gγ$_{30A}$)

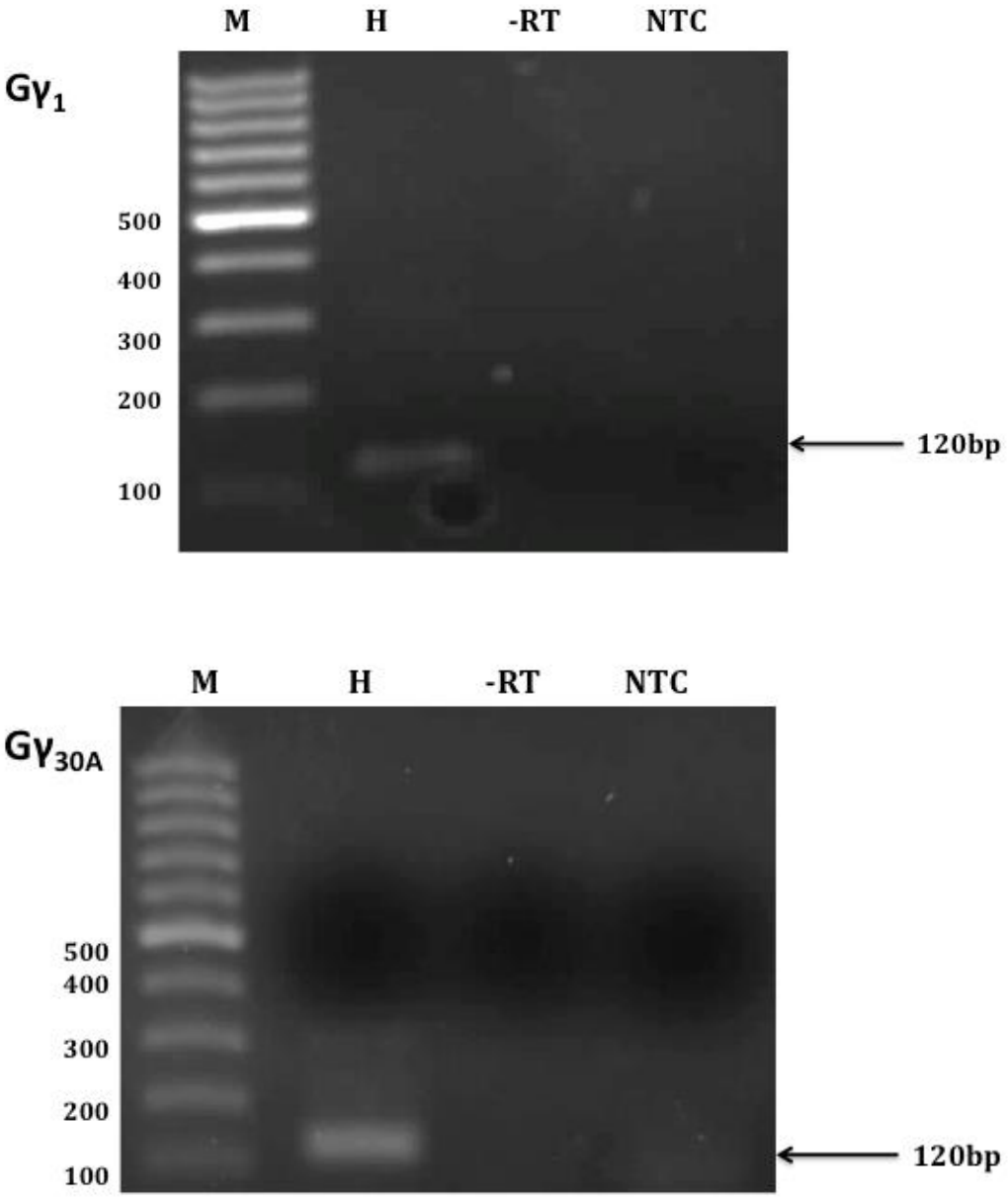


Figure 3. Expression analysis of Gγ genes (Gγ$_1$ and Gγ$_{30A}$) in the head of adult *Drosophila* using reverse transcriptase chain reaction (RT-PCR)

Target genes are denoted on the left of the figure and the amplification product size on the right, expressed in base pairs (bp). RNA was extracted from the head of the flies and this is identified at the top of each figure as follows: M: 100bp ladder; H: head; -RT (minus reverse transcriptase); NTC (No template control).

Appendix 4.2

Wild-type flies were exposed to ethanol either once (acute response in naïve flies) or three times (acute response in chronic flies). mRNA measurement for G proteins were carried out 1 h after the last exposure. Ethanol treatment did not induce significant changes in any of the Gα subunits (G_s, G_o, G_f and cta) compared to control (naïve flies) (Figure 1). Variations were observed within the population of wild-type flies and each independent experiment.

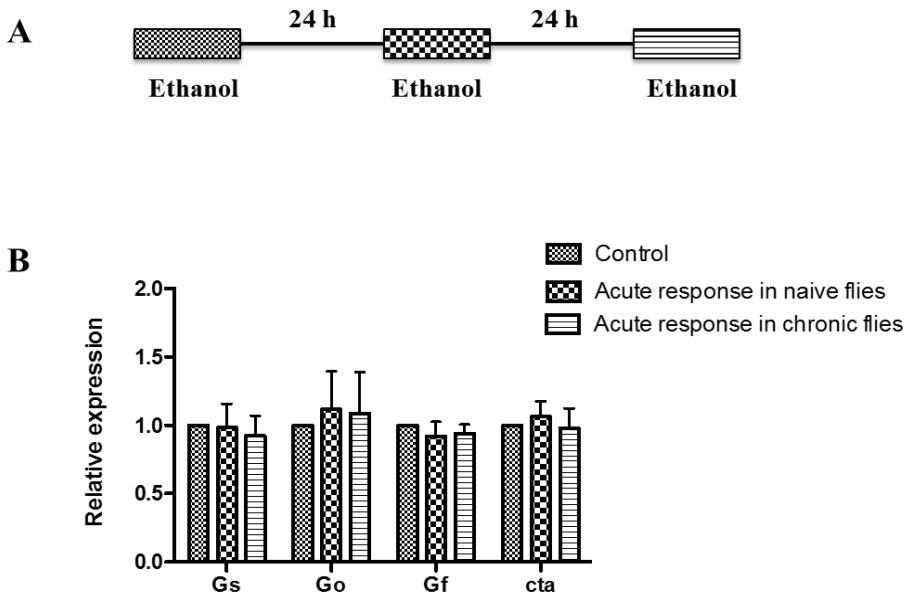


Figure 1. Effect of ethanol treatment on Gα genes in *Drosophila*'s head

(A) Ethanol exposure protocol which induces rapid tolerance to alcohol. Flies were exposed three times to 500µl of 100% ethanol for 30 min each. A 24 h recovery period was allowed between each ethanol treatment. (B) Effect of ethanol administration on G protein subunit mRNA expression in *Drosophila's* head. Total RNA was extracted from the head of control (flies that have never been exposed), one exposure (acute response in naïve flies) and three exposures (acute response in chronic flies). Relative expression in mRNA were quantified relative to control (naïve flies) and normalized to an internal control, β-actin. Error bars represent ± Standard Error of the Mean (SEM) (n= 4 independent experiments, n=5 female flies, Student's T-test, $P>0.05$).

Appendix 5.1

Sequencing of RT-PCR product of G_i subunit (Below) was carried out to validate the observed results obtained from real-time polymerase chain reaction. The sequences were verified by a BLAST search, which matched 100% of the deposited G_i.

Sequences:

GiF_premix -- 20.88 of sequence

AGATCGCATGATTGAGTCCCTGAAGCTGTTCGATTCCATCTGCAACTCCAAG
TGGTTTGTGGACCTCAA

GiR_premix -- 27.85 of sequence

ATCATGCGATTCATCTCTTCGTCCTCGGCCAAAACTAGATCGTAACCTGATA
GCGCGAC

Appendix 6.1

The mechanism by which ethanol-induced treatment results in G protein gene expression changes remains unknown. microRNAs (miRNAs) are thought to play a significant roles in mediating the effects of ethanol (Miranda *et al*., 2010). They are highly abundant in the brain and are known to be involved in many biological processes, including brain development (Cheng *et al*., 2009). Changes in miRNAs have been implicated in the development of ethanol-induced tolerance, a crucial component of alcohol addiction (Miranda *et al*., 2010, Pietrzykowski and Treistman, 2008). Altered expression of some miRNAs could be due to ethanol altering the activities of various signal transduction pathways, including tyrosine and MAP kinases or due to epigenetic changes (Miranda *et al*., 2010). Given that miRNAs potentially suppress the expression of their target genes, ethanol can cause simultaneous up regulation of some miRNAs while down regulating others.

MicroRNAs are small non-coding RNAs (~23nt) that are thought to act as posttranscriptional modulators of gene expression, by binding to the 3'-untranslated regions (UTRs) of their target genes resulting in either the suppression of translation or degradation of mRNA transcripts or both (Fillipowicz *et al*., 2008, Lewohl *et al*., 2011). Recently, it has been reported that miR382 expression is affected by ethanol exposure. It is linked with *ΔfosB*, which is a nuclear factor associated with the response of addictive drugs (Li *et al*., 2013).

In *Drosophila*, TargetScanFly (Ruby *et al*., 2007) searches for both conserved and non-conserved sites through seed sequence complementarity between the miRNA and 3'UTR (Lucas and Raikhel, 2013) was carried out. Using the TargetScan to analyse the mRNA sequences of the G proteins, which have been observed to be reduced during repeated ethanol treatment, G_i was identified to contain the binding consensus sequence of the known miRNA miR-8, whereas G_q contains the known miRNA miR-315 (See

below). In future, the predicted miRNA targets could be used to study the mechanism of the changes in gene expression observed using qRT-PCR. This could be achieved by transfecting the *Drosophila* S2 cell line with the synthetic miR-8 or miR-315 and the expression of each G_i or G_q would be determined using qRT-PCR respectively.

An image of the predicted miRNA target for G_i (G-ialpha65A) subunit using TargetScan is shown below. miR-8 was predicted for G_i gene (denoted by the red circle).

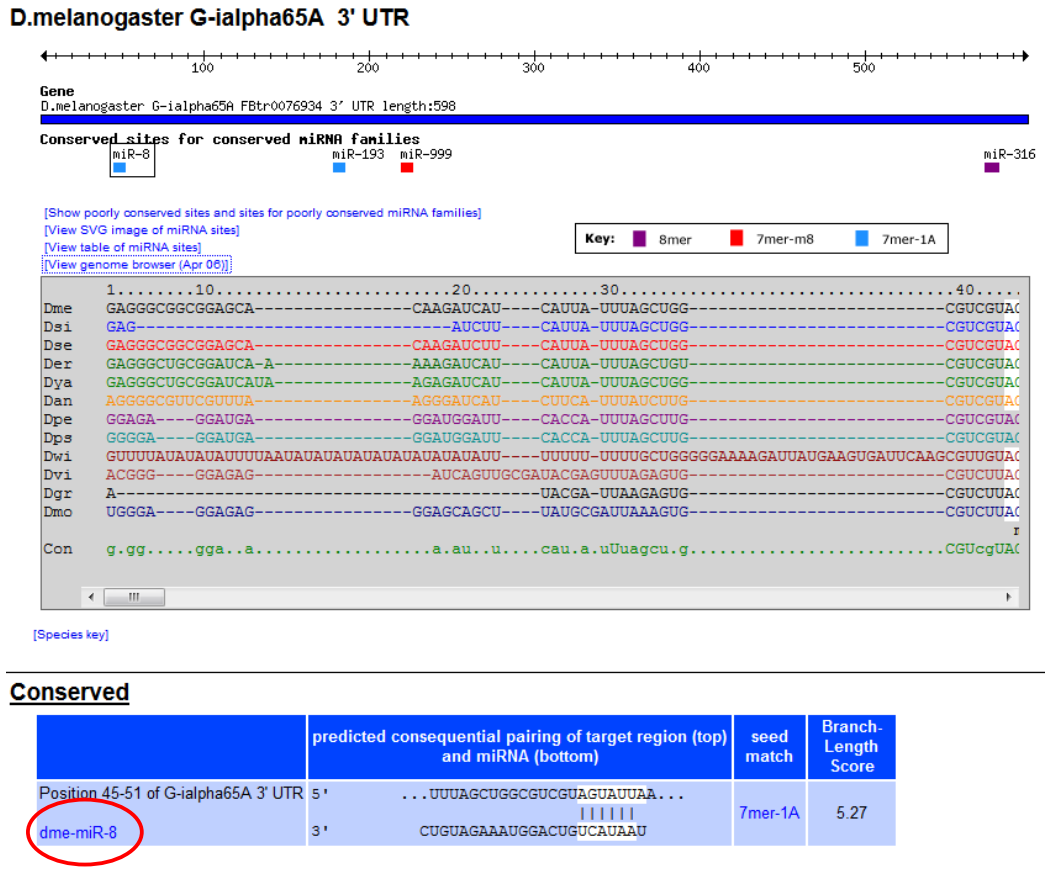


Figure 1. Output of miRNA search on TargetScan (http://www.targetscan.org/fly_12/) using G-ialpha65A as a target gene. The identified miRNA is circled in red.

The predicted miRNA target was obtained by enterning the Flybase symbol (CG10060) in the search box. The identified miRNA (dme-miR-8) is circled in red.

An image of the predicted miRNA target for G_q (Galpha49B) subunit using TargetScan is shown below. miR-315 was predicted for G_q gene (denoted by the red circle).

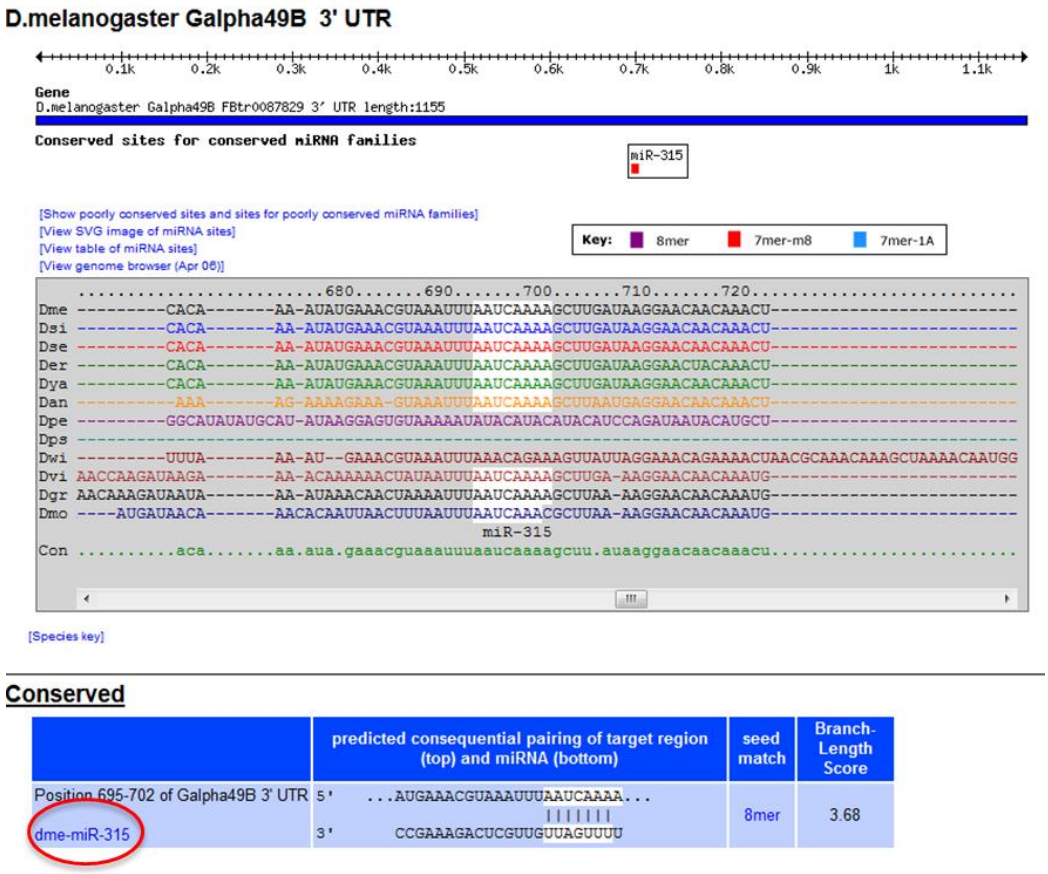


Figure 2. Output of miRNA search on TargetScan (http://www.targetscan.org/fly_12/) using Galpha45B as a target gene. The identified miRNA is circled in red.

The predicted miRNA target was obtained by enterning the Flybase symbol (CG17759) in the search box. The identified miRNA (dme-miR-315) is circled in red.