The Role of Gamma-Aminobutyric Acid B Receptors in Alcohol Related Behaviours in *Drosophila* Models of Ethanol Tolerance and Preference

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Abstract

Alcohol is one of the most widely used and socially accepted psychoactive substances in the world, and its misuse was accountable for 3.3 million alcohol related deaths in the world in 2015. Whilst it is known that ethanol enhances the actions of the $GABA_B$ receptor, the role of the stimulation of this receptor in inducing acute and chronic effects, remains to be fully understood. The fruit fly, *Drosophila melanogaster,* offers the possibility to investigate behaviours such as preference and tolerance to alcohol, and to challenge them with pharmacological agents. In this study, the $GABA_B$ receptor agonist (SKF 97451) and antagonist (CGP 54626) were used to challenge the development of tolerance and the onset of preference to alcohol in wild type flies and in mutant lines with putative disruptions of $GABA_B$ receptor 1 or 2 subunit genes. Both compounds were able to alter the onset of tolerance measured as the time needed for half of a set of flies to be sedated by alcohol. Additionally, both drugs affected the preference developed by the flies towards alcohol containing food measure in a capillary feeder assay. The $GABA_B$ receptor mutant flies provided further evidence that the receptor is involved in the behavioural process studied. Overall the results indicate that the $GABA_B$ receptors are indeed part of a complex mechanism that result in alcohol induced behavioural changes. The data supports the usefulness of the *Drosophila* model and the need of further investigations into the $GABA_B$ receptor and to other potential pathways and mechanisms that could be contributing to the onset of such behaviours.

Declaration

This thesis is a presentation of my own original work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was done under the guidance of Dr Stefano Casalotti and Dr Samir Ayoub at the University of East London, Stratford, London.

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In my capacity as Director of Studies for the candidate's thesis, I certify that the above statements are true to the best of my knowledge.

Name: **Dr Stefano Casalotti**

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Abbreviations

%	Percent
ANOVA	Analysis of Variance
bp	Base Pairs
CAFE	Capillary Feeder
cDNA	Complementary Deoxyribonucleic Acid
CGP 54626	$GABA_B$ Receptor Antagonist
CO_2	Carbon Dioxide
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EtOH	Ethanol
g	Grams
GABA	γ- aminobutyric acid
$GABA_A$	γ- aminobutyric acid A
$GABA_B$	γ- aminobutyric acid B
GBP	Great British Pounds (Stirling)
GPCR	G-Protein Coupled Receptors
h	Height
KD	Knock Down
KO	Knock Out
MCH	4-Methylcyclohexanol
mm	Millimetres
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
nM	Nanomolar
nmol	Nanomole
ø	Diameter
ºC	Degree Celsius
OCT	3-Octanol
PAMS	Positive Allosteric Modulators
PI	Preference Index
R1	$GABA_B$ Receptor 1 Subunit
R2	$GABA_B$ Receptor 2 Subunit
RNA	Ribonucleic Acid
RNA	Ribonucleic Acid
SEM	Standard Error of Means
SKF 97541	$GABA_B$ Receptor Agonist
ST50	Time to 50% of flies sedated
w/v	Weight/Volume Percent
µg	Microgram
µL	Microlitre

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I am thrilled to be able to thank everybody who has made this thesis possible. I would firstly like to acknowledge and recognise my Director of Studies, Dr Stefano Casalotti. I would like to express my deepest gratitude to Dr Casalotti for the patience and time he has given me throughout this project and for being there to provide the assistance and support whenever I needed it. I would not be in the position I am without him; Stefano is a true inspiration to me.

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I would like to move on to thank my Postgraduate Research peers, my university family! The support and laughs that you have all provided to keep me smiling through the ups and downs, I am very grateful!

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Chapter 1: Introduction

1.1. Alcohol Addiction

Researching the acute and chronic effects of alcohol addiction has been a priority for researchers for many years because alcohol abuse has been identified as a problem worldwide. An estimated 3.3 million people die annually as a result of alcohol abuse and alcohol related incidences, equating to 5.9% of all deaths worldwide (World Health Organistation [1] 2015).

Although the term addiction is controversial, throughout this thesis 'addiction' will refer to the way an individual subject shows a positive preference or positive tolerance as a result of the intake and or exposure to alcohol (Volkow *et al.* 2016). The words alcohol and ethanol will be used interchangeably throughout this thesis and when alcohol/ethanol is used when describing experimentations with the *Drosophila melanogaster*, it will always be referring to ethanol (EtOH) (Figure 1.1)

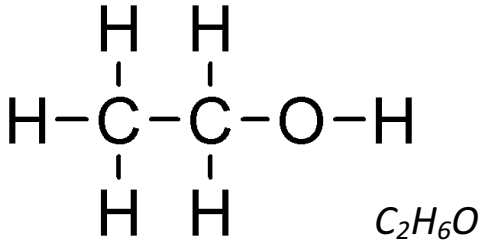


Figure 1.1: The chemical structure of ethanol. Image created with ChemDraw Direct version 1.5.1.190.

1.2. Alcohol Users

Alcohol Concern (2017) state that 16% of men and 9% of women who drink alcohol do so at least five of the seven days per week. It was reported that more than 9 million people in the UK drink more than the recommended daily limits. Of these 9 million alcohol users, 34% of men and 28% of women drank more than the recommended units per week (14 units per week) as dictated by the National Health Service (NHS) (National Health Service 2017).

1.2.1. Alcohol Related Deaths

The Office for National Statistics (ONS) reports that in the year 2015, in the UK there were 8,758 alcohol related deaths; this corresponds to a death rate of 14.2

deaths per 100,000 people (Figure 1.2/Appendix 1) (Office for National Statistics 2017).

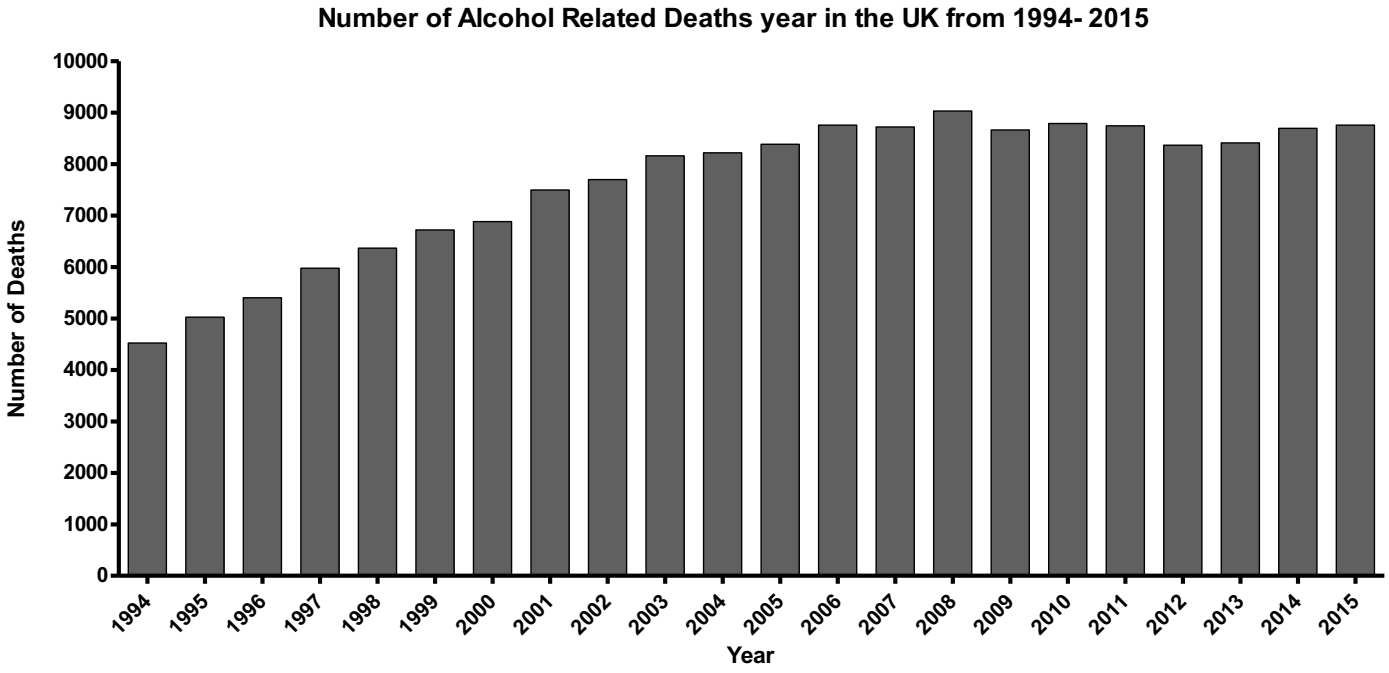


Figure 1.2: A graph to highlight the number of alcohol related deaths in the UK from 1994 to the year 2015. Figure created based upon data within a publication produced by the Office for National Statistics (2017). See Appendix 1 for raw data.

ONS's definition of an 'alcohol related death' includes "…underlying causes of death regarded as those being more directly due to alcohol consumption" (Office for National Statistics 2017). This report by the ONS is considered unbiased and a good true indication of an alcohol related death due to using a strict International Classification of Diseases (ICD) coding system (Table 1.1). It is also important to note that this definition and all statistics published from the OFN publication titled '*Alcohol-related deaths in the UK: registered in 2015'* excluded external causes of death as a result of alcohol related accidents, such as road traffic and other alcohol related accidental deaths as a result of intoxication from alcohol. This therefore gives a true reflection on the effect of alcohol on the human body.

Table 1.1: A table to portray the International Classification of Disease codes along with representing description of conditions.

ICD Code	Description of Condition
F10	Mental and behavioural disorders due to use of alcohol
G31.2	Degeneration of nervous system due to alcohol
G62.1	Alcoholic polyneuropathy
I42.6	Alcoholic cardiomyopathy
K29.2	Alcoholic gastritis
K70	Alcoholic liver disease
K73	Chronic hepatitis, not elsewhere classified
K74	Fibrosis and cirrhosis of liver (Excluding Biliary Cirrhosis)
K86.0	Alcohol induced chronic pancreatitis
X45	Accidental poisoning by and exposure to alcohol
X65	Intentional self-poisoning by and exposure to alcohol
Y15	Poisoning by and exposure to alcohol, undetermined intent

Table modified from Office for National Statistics (2017). See Appendix 2 for raw data.

1.2.2. Alcohol Related Death Statistics

From the year 1994 to 2015, it can be said that the age group including 50 to 54 year old people from the UK have the highest number of alcohol related deaths. Excluding the 15 to 19 year old category due to 60% of the age group not being legal to purchase alcohol in the UK, the lowest number of alcohol related deaths is the 20-24 year old category (Figure 1.3/Appendix 2). It is at present unconfirmed, but it is assumed by the ONS that this is due to this age category being of legal age to drink alcohol for the least period of time and therefore having the least amount of time to develop alcohol related health conditions (Office for National Statistics 2017).

Figure 1.3: A graph to highlight the number of alcohol related deaths in the UK from 1994 to the year 2015 categorised by age. Figure created based upon data within a publication produced by the Office for National Statistics, 2017. See Appendix 1 for raw data.

1.2.3. Alcohol and UK Emergency Services Statistics

Alcohol use is a huge burden to the society within the UK. The National Health Service (2017) estimates that alcohol abuse costs the emergency services around £21 billion GBP per year. The breakdown highlights that £3.5 billion GBP is spent by the NHS, £11 billion GBP is used to tackle alcohol-related crime and a total of approximately £7.3 billion GBP is due to loss of working hours (National Health Service 2017, Office for National Statistics 2017).

Within England, the Office for National Statistics (2017) states that alcoholic liver disease is responsible for the highest number alcohol related deaths amongst all males and females in England with 63.3% of all deaths. Fibrosis and cirrhosis of the liver is the second largest ailment, accountable for 22.6% of all deaths, followed by mental and behavioural disorders due to alcohol use answerable for 7.7% of all alcohol related deaths (Appendix 2).

It is well documented that alcohol is one of the most addictive and most harmful psychoactive substance (Nutt *et al.* 2010). Worryingly, whilst we are aware of the addictive properties of alcohol, there is at present a real absence of understanding of the development of addictive mechanisms at the cellular and molecular level. More work needs to be undertaken and much of this research will require the use of animal models to identify the key molecular players and potential targets of pharmacological intervention that would then help to combat the number of alcohol related deaths, and to alleviate the burden and strain that alcohol use is having on the UK emergency services.

1.3. Animal Models

In recent years, animal models have been documented as being of extreme importance for identifying the key mechanisms induced by acute and chronic consumption of alcohol. Mammalian studies have provided a good foundation into molecular mechanisms regarding ethanol consumption but there are certain ethical limitations that prevent unrestricted testing. Fadda *et al.* (2003), Vlachou *et al.* (2011), Crabbe (2014), Hwa *et al.* (2014), Meye *et al.* (2016) have all described successes when using the rodent as a model to study the mechanisms of alcohol addiction. However, the *Drosophila melanogaster* has been identified as being a better and truly unbiased model for studying the acute and chronic effects of alcohol abuse (Devineni and Heberlein 2009). The underlying fundamental when using any animal model to replicate a human disease, is to ensure simplicity, reproducibility and accuracy with respect to human behaviours and the *Drosophila* in this instance is a highly valuable model when used to study alcohol addiction (Kaun *et al.* 2012).

1.4. γ-Aminobutyric Acid

It is known that γ-Aminobutyric Acid (GABA) is an inhibitory neurotransmitter in the central nervous system and its principle role of action is in reducing neuronal excitability via the inhibition of nerve transmissions within the brain (Bowery *et al.* 2002, Watanabe *et al.* 2002, Manev and Dzitoyeva 2010, Benarroch 2012). There are two classes of GABA receptor; γ-Aminobutyric Acid A receptors ($GABA_A$) which are a ligand-gated ion channel complex that upon ligand binding, open a chloride channel, and γ-Aminobutyric Acid B receptors ($GABA_B$) which are G-protein coupled receptors responsible for modulating calcium and potassium

channels which in turn elicit both pre-synaptic and post-synaptic inhibitions (Figure 1.4).

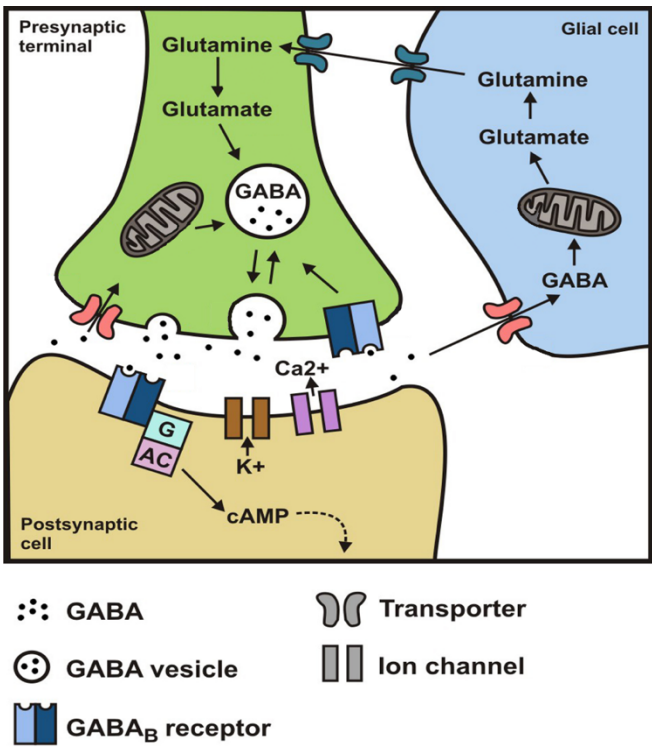


Figure 1.4: Synthesis, release, binding and reuptake of GABA in a synapse. Image taken and modified with permission from Hyland and Cryan (2010).

1.4.1. GABA$_B$ Receptors as a Potential Target for Alcohol Addiction

Multiple researchers using different animal models have been successful in demonstrating a reduction in alcohol consumption by targeting the GABA$_B$ receptors with agonist ligands; Hwa et al. (2013) have used GABA$_B$ positive allosteric modulators (PAMS) to decrease voluntary ethanol intake in mice while Dzitoyeva et al. (2003) have documented that gene silencing of GABA$_B$ receptors in *Drosophila* leads to a reduction in the behaviour-impairing effects of ethanol. This therefore suggests a putative role for the *Drosophila* GABA$_B$ receptors in alcohols mechanism of action.

As part of other addiction studies, it was identified by Fadda *et al.* (2003), Vlachou *et al.* (2011) that when stimulating the GABA$_B$ receptor with an agonist ligand, the addictive behaviours associated with both nicotine and cocaine are suppressed, further reinforcing that GABA$_B$ receptors play a role in addiction behaviours of addictive compounds including alcohol.

The work detailed within this thesis builds upon previously published works on identifying the role of the $GABA_B$ receptor in establishing and maintaining alcohol like addiction behaviours in the *Drosophila melanogaster*.

1.5. Overall Aim

The overall aim of this thesis is to expand on current knowledge and to provide a better understanding on the role of $GABA_B$ receptors with respect to modulating alcohol induced behaviours.

1.5.1. Objectives

- To refine and continue to develop the *Drosophila melanogaster* as a suitable model to study the effects of ethanol induced addiction like behaviours.
- To determine whether flies in which the $GABA_B$ genes have been altered behave differently in response to alcohol
- To investigate if administration of a $GABA_B$ receptor agonist or antagonist can affect tolerance or preference to ethanol development.
- To determine if a developed preference or tolerance to ethanol can be reversed by the administration of a $GABA_B$ receptor agonist or antagonist.

1.5.2. Research Hypothesis

It is hypothesised that the $GABA_B$ receptor plays a modulatory role in response to alcohol, initiating and/or maintaining tolerance and preference behaviours associated with addictive traits. It is further hypothesised that the $GABA_B$ receptor is responsible or at the very least part of a pathway that is responsible for the onset and maintenance of ethanol induced behaviours.

Chapter 2: Materials and Methods

2.1. *Drosophila* Husbandry

Drosophila were raised and assayed whilst housed in an incubator at 25°C unless stated otherwise. *Drosophila* stocks were housed at 18°C to slow growth and keep metabolism low. At both temperatures, flies were kept in incubators that had a twelve-hour light/dark cycle with between 60-80% relative humidity.

Drosophila were placed into large 220g round bottom bottles measuring 59mm (ø) x 130mm (h) (Genesee Scientific, USA) or smaller vials measuring 25mm (ø) x 95mm (h) (Dutscher, France). Dry pre-mixed *Drosophila* food (Phillip Harris, UK) was prepared according to manufacturer's guidelines and was placed into vials/bottles approximately 10-15mm deep. Cotton or sponge like vial/bottle plugs were used to prevent escapees of flies (Genesee Scientific, USA and Dutscher, France).

2.2. *Drosophila* Strains

Drosophila strains used within this project include Wild type Canton-S and mutant $GABA_B$ R1 and $GABA_B$ R2 knock out flies (Table 2.1). All *Drosophila* strains were obtained from Bloomington *Drosophila* Stock Centre (Indiana, USA).

Table 2.1: Summary of *Drosophila* lines used

Fly Line	BDSC ID	Information
Wild Type (Canton-S)	64349	Wild type *Drosophila* containing no genetic modifications.
$GABA_B$ Receptor 1 Knock Out	44860	Contains a transposon insertion (Mic cassette) in the $GABA_B$ R1 gene. Insertion location between nucleotide numbers 15,033,257 and 15,033,257 on the second chromosome.
$GABA_B$ Receptor 2 Knock Out	59503	Contains a transposon insertion (Mic cassette) in the $GABA_B$ R2 gene. Insertion location on the between nucleotide numbers 21,773,501 and 21,773,501 on the third chromosome.

2.3. *Drosophila* Standardisation

All *Drosophila* used were collected when aged between 1-5 Days after eclosion to minimise assay variance between age groups. All initial control experiments were carried out on both male and female flies to explore the differences in preference and tolerance. In later stages when there were pharmacological interventions, experimentations were conducted on only male flies for all fly lines. Flies were sorted into vials by sex under a stereo microscope whilst under light carbon dioxide (CO_2) anaesthesia.

2.4. Behavioural Assays

In order to measure behavioural effects as a result of exposure to ethanol, various assays were used to identify the level of tolerance and preference to ethanol (Figure 2.1). The tolerance assay was used to measure how the response to ethanol changed over a set period of time as described by Maples and Rothenfluh (2011). A consumption based capillary feeder (CAFE) was used to evaluate the preference to ethanol containing food or non-ethanol containing food as first described by Ja *et al.* (2007) and further optimised and refined by Devineni and Heberlein (2009). Preference was further explored in a conditional preference assay by associating ethanol with neutral smelling odours through 'training' exposures and allowing free movement during a 'test' session in which flies made a choice for the odour in absence of ethanol, following a methodology similar to that by Tempel *et al.* (1983), Simonnet *et al.* (2014).

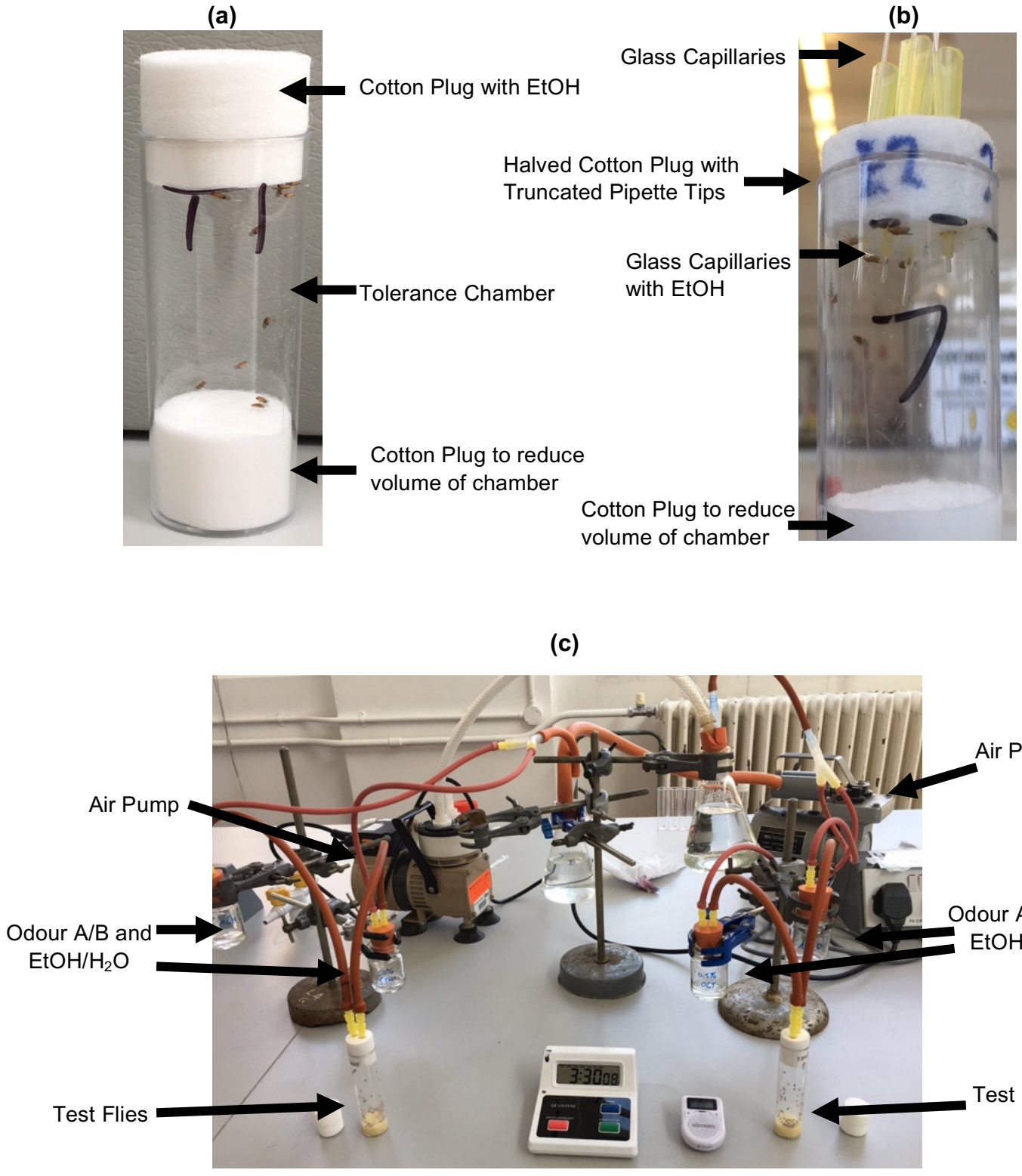


Figure 2.1: Pictures highlighting the behavioural assays used. **(a)** Tolerance Assay, **(b)** Preference Assay and **(c)** Conditional Preference Assay**.**

2.4.1. Tolerance Assay

Sensitivity to ethanol is more commonly descibed as 'tolerance', and refers to "…the need of an individual either sub-conscienсsly or consciencly, engageing in

a particular behaviour at a relatively greater rate/level than previously, to achieve a greater effect than before" as described by Sussman and Sussman (2011).

Tolerance assays as described by Scholz *et al.* (2000), Maples and Rothenfluh (2011) were modified to meet personal and laboratory equipment specifications and were conducted using a method as detailed below (Figure 2.2).

Ten male/female flies (sex is dependent on assay and is stated within results legend) were anesathised and sorted into sexes the day before assay was due to begin to allow recovery from CO_2 anaesthsia (Step 1 in Figure 2.2). At the time of assay, flies were transferred with no anaesthatisation to the 'tolerance chamber' (Step 2 in Figure 2.2).

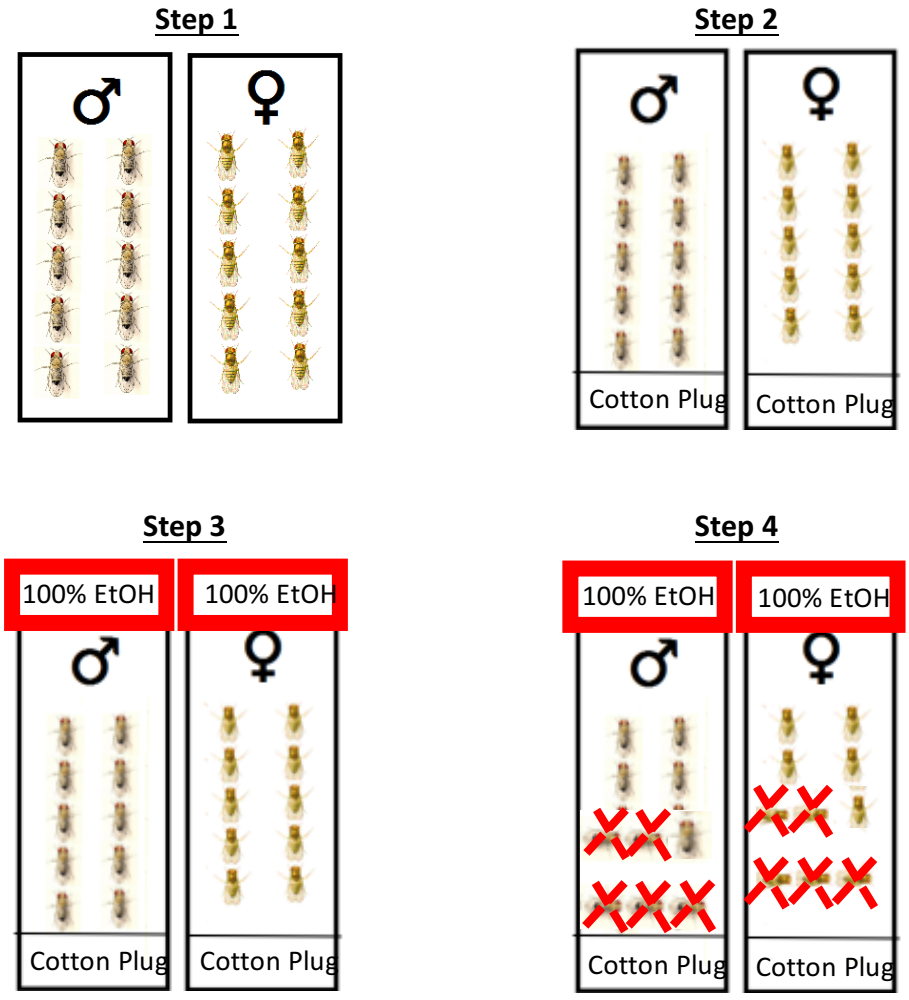


Figure 2.2: Pictorials highlighting the key stages in a tolerance assay.

Step 1: *Image shows sorted test flies recovering from C0$_2$ anaesthesia (for at least 12 hours) housed in an empty vial ready for transfer to tolerance chamber.* ***Step 2:*** *Flies are transferred to tolerance chamber with no anaesthsia.* ***Step 3:*** *500µl of 100% EtOH is*

added to a cotton plug and placed onto tolerance chamber vial. ***Step 4:*** *100% EtOH exposure occurs until ST50 (Time to 50% of flies sedated) occurs.*

This modified vial contained a cotton vial plug placed into the bottom of the tolerance chamber to reduce the volume of the chamber and thus increase ethanol concentrations and to decrease sedation times. The addition of the cotton vial plug to the tolerance chamber reduced the total air volume of 91.40cm^3 and the flying height to 55mm, compared with the standard total air volume of 237.50cm^3 and flying height of 95mm. This is a reduction of 61.5% air volume and 42% flying height compared to the standard vial.

Flies once transferred, were exposed to 500µl of 100% ethanol by placing this liquid onto the centre of a cotton vial plug and inverting it into the vial (Step 3 in Figure 2.2). Flies were left to be exposed until 50% of the total number of flies were sedated; this time point was defined as ST50 (Time to 50% of flies sedated). Flies were tapped three times onto the lab bench once per minute to disorientate and startle the *Drosophila* and to knock them to the bottom of the vial (Step 4 in Figure 2.2). Flies were observed for 10 seconds and the number of stationary flies was recorded for each minute. It was important to determine and standardise what 'stationary' and 'sedated' was. Sedation/stationary for this assay was determined as upon being knocked to the bottom of the vial, flies were unable to upright themselves within the 10 second observation time. Any leg movement or vibrating of the wings were ignored and if the flies were still upon their backs with the inability to self right, they were recorded as sedated.

Tolerance was measured over a period of three days and the ST50 was measured for each vial for each day (Figure 2.3). Tolerance is identified as being an increase in ST50 over consecutive days of exposure with ethanol having a reduced effect on sedation and subsequently resulting in an increased ST50 than for prior ethanol exposures (Scholz *et al.* 2000, Atkinson 2009, Maples and Rothenfluh 2011, Chan *et al.* 2014)

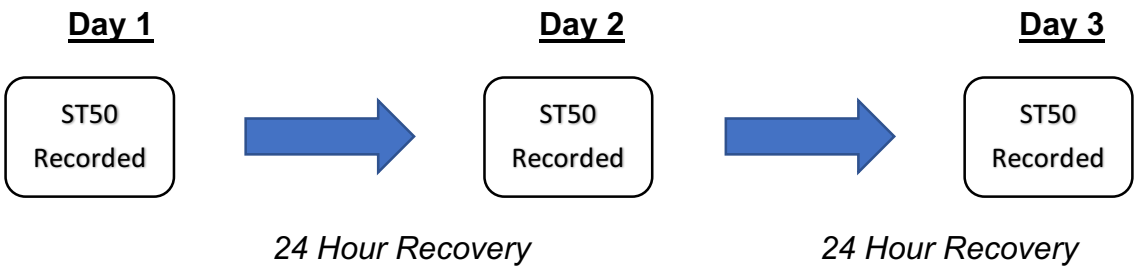
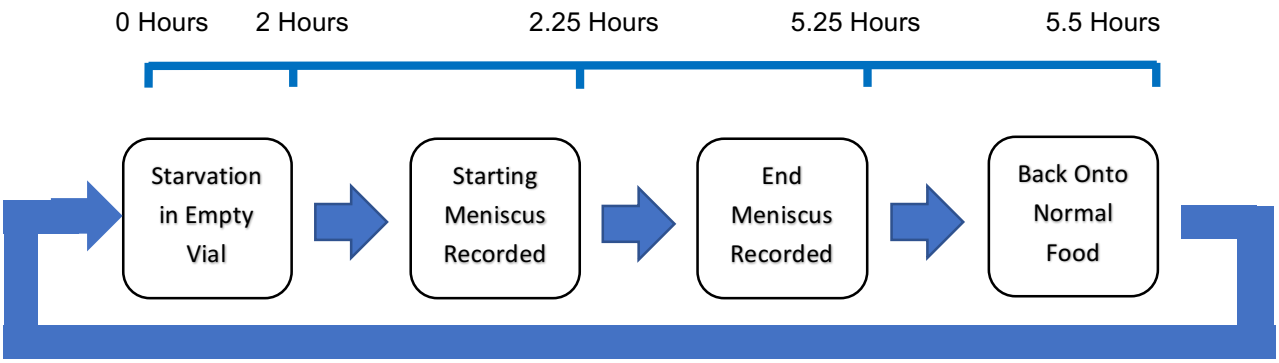


Figure 2.3: A flow chart highlighting the tolerance assay schedule

2.4.2. Preference to Ethanol Assay (CAFE Assay)

The CAFE assay is a tool that allows consumption of liquid foods to be measured in real-time. The capillaries used (Jaytec, UK), are 59mm in length and have a total volume of 5µl and consumption of liquid foods is clearly measurable by viewing the decreasing capillary meniscus. The consumption of ingested liquid foods can be measured in a group or individuals *Drosophila* for time periods ranging from one minute to an entire lifespan. This project used the CAFE assay for a period of 3 hours once per day after a starvation period of approximately 2 hours prior to each assay. This was to ensure that consumption was high enough to be measured (Figure 2.4). The CAFE assay is an extremely useful tool used by many to measure food consumptions, preferences to different drugs, substances, foods, environments or even treatment regimens to name a few (Ja *et al.* 2007, Devineni and Heberlein 2009, Pohl *et al.* 2012, Peru *et al.* 2014).



24 Hour Recovery on Standard Solid Food and then next day is started as above

Figure 2.4: A flow chart highlighting the preference assay schedule for one day.

The CAFE assay apparatus consisted of a standard plastic vial (as described in chapter 2.1.1.) that had been altered to provide 2 chambers (Figure 2.5); one chamber for Drosophila, and one chamber to provide humidity separated with a

halved cotton vial plug. Four capillaries were used to provide the liquid food to the *Drosophila* and were held in place by placing the capillaries into truncated 200µl pipette tips as explained by Ja *et al.* (2007), Devineni and Heberlein (2009) (Figure 2.5). Flies had a flying height in chamber 1 of 35mm and the second chamber had a water saturated Kimwipe (Kimtech Science, USA) to help raise and maintain humid air in the vial and prevent dehydration of the *Drosophila*. Prior to the CAFE assay, flies were sorted into groups (depending on assay requirements) of 5 or 10 flies by CO_2 anaesthetisation and were placed into the assay vial. Vials were kept at 25°C with a relative humidity of between 60-80%, except when the meniscus level of the capillaries was being recorded. A replicate CAFE vial was always used during experimentations to record evaporation and subtract this from all capillary meniscus readings.

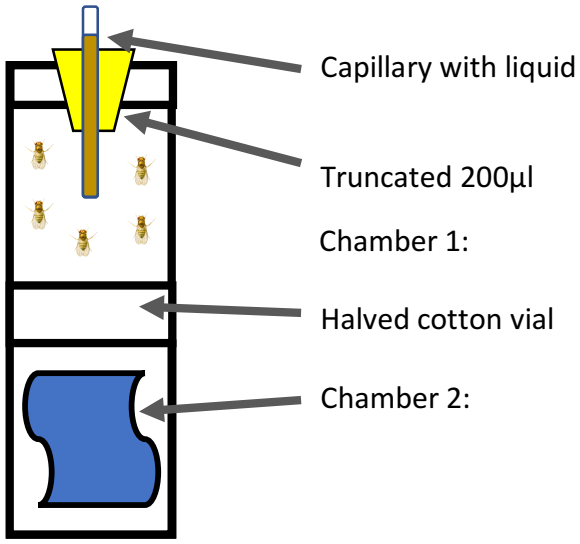


Figure 2.5: Schematic of the CAFE assay vial set up with one capillary shown

During all CAFE assays, there were two different liquid foods used in a total of four capillaries per vial (Figure 2.6). The standard liquid food was composed of 5% yeast extract and 5% sucrose solution dissolved in distilled water and the ethanol containing food was composed of 5% yeast extract and 5% sucrose solution dissolved in 15% ethanol. Preference was measured by providing two of each capillary and allowing consumption for a set period of time dependent on assay. Ja *et al.* (2007), Devineni and Heberlein (2009) illustrate how the preference index was measured for all CAFE assays during this project:

$$\textbf{Preference Index: } \frac{\text{Ethanol Containing Food Consumed} - \text{Non-Ethanol Containing Food Consumed}}{\text{Total Volume of Food Consumed}}$$

The preference index (PI) is a way of standardising the level of preference that the *Drosophila* have for one food over another (Peru *et al.* 2014). The PI is scored from -1 whereby there is a 100% negative preference to a food, to +1 whereby there is a 100% positive preference to the experimental food in question (Ja *et al.* 2007, Devineni and Heberlein 2009, Pohl *et al.* 2012, Peru *et al.* 2014).

Figure 2.6: CAFE vial highlighting the capillary containing liquid foods. Image used with permission from Devineni and Heberlein (2009).

2.4.3. Conditional Preference Assay

The conditional preference assay was built and designed around a 'Y maze' design and concept. The assay consisted of 2 parts: 1) a conditioning/ odour association training phase, and 2) an unbiased free choice preference test (Figure 2.1 and 2.7). This assay was configured to allow the *Drosophila* to make an unbiased choice to show preference to ethanol or not. Two neutral smelling odours, 4-Methylcyclohexanol (MCH) and 3-Octanol (OCT) (Sigma, UK) were used as association odours to either 50% EtOH or a H_20 control.

Following methodologies similar to those used by Tempel *et al.* (1983), Masek and Heisenberg (2008), Yarali *et al.* (2009), Simonnet *et al.* (2014), a Y maze test vial and training apparatus was constructed (Figure 2.1 and 2.7). Part 1 of the assay was the conditioning/training phase of the assay. *Drosophila* were first placed into vials with modified cotton vial plugs. Holes were placed into the vial

plug to allow 200µl pipette tips to be placed through the holes, allowing odour to be pumped into the vial via rubber hosing. An air pump pumped air at a rate of 21.4 litres per minute into two glass bottles filled with a 5ml total of either 1% MCH or 0.5% OCT dissolved in dH_2O and 50% EtOH or dH_2O (See Step 1&2 in Figure 2.7). Flies were exposed to the first odour and EtOH/dH_2O for ten minutes and were then given the second odour and EtOH/dH_2O followed by a one hour recovery period to rest the olfactory systems. The training occurred a total of three times. Vials were kept at 25°C with a relative humidity of between 60-80%, except when the training exposures were taking place.

The Y maze preference test was conducted 24 hours after the last training session (See Step 3 in Figure 2.7). Flies were given an unbiased choice of moving through the Y maze arms to the OCT odour or the MCH odour and the number of flies making either choice was recorded. In separate experiments ethanol was associated with different odours, to ensure that the preference was for EtOH and was not dependent on the associated odour instead. Flies had free movement in the Y maze for 3 minutes to make their choice.

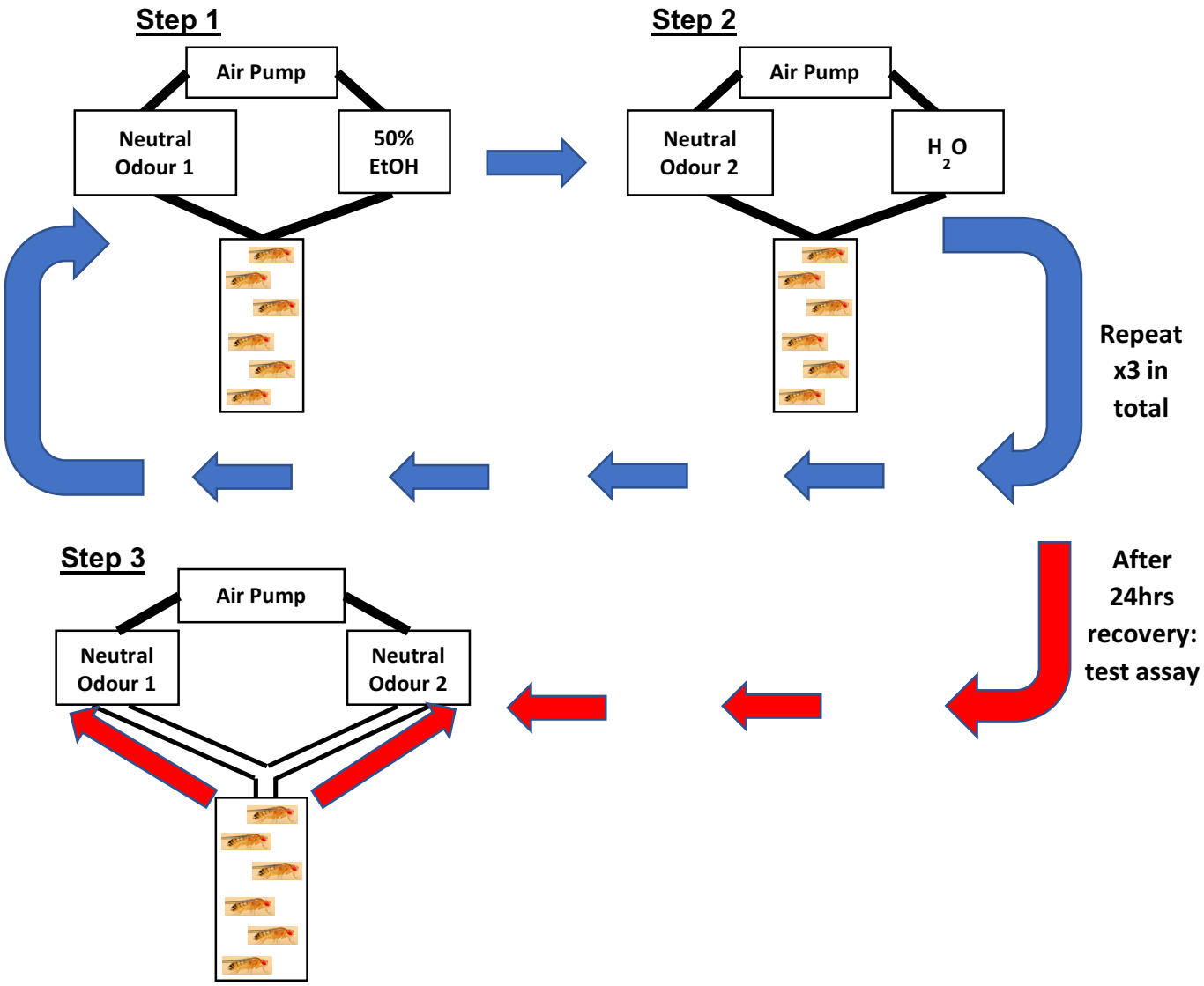


Figure 2.7: A schematic showing training and test schedule for conditional preference assay.

Preference was measured in a similar way as described in chapter 2.2.6 based upon the preference index used by Ja *et al.* (2007), Devineni and Heberlein (2009). The conditional preference for ethanol was calculated in the following way:

$$\textbf{Preference Index:} \frac{\text{Ethanol Associated Odour} - \text{Non-Ethanol Associated Odour}}{\text{Total Number of Flies That Made a Choice}}$$

It is important to note that only flies that made a choice to move into one of the Y arms were included within the preference index. Flies that remained in the holding vial at the bottom or remained stationary in the entrance neck of the Y maze were not included to remain true to unbiased preference.

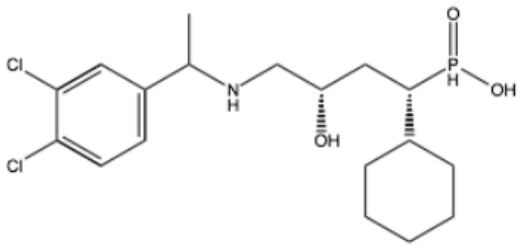
2.5. Drug Administration

Known selective $GABA_B$ receptor drugs were sourced from Tocris Bioscience, part of the BioTechne, UK brand. An angonist (SKF 97541) and antagonist (CGP 54626) were sourced and are detailed within table 2.2 and figure 2.8. Drugs were administered within the *Drosophila's* ready mixed standard food. The water normally used to prepare the flies ready mixed dry food, was substituted for the drug dissolved in distilled water at the relevant concentration.

Table 2.2: A table documenting properties of $GABA_B$ receptor ligands

Drug Name	Chemical Name	Chemical Formula	Molecular Weight	Biological Activity
CGP 54626	[*S*-(*R**,*R**)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid	$C_{18}H_{28}Cl_2NO_3PHCl$	444.76	Selective $GABA_B$ receptor antagonist
SKF 97541	3-Aminopropyl(methyl)phosphinic acid	$C_4H_{12}NO_2P$	137.12	Very potent selective $GABA_B$ receptor agonist

(A) CGP 54626 antagonist ligand



(B) SKF 97541 agonist ligand

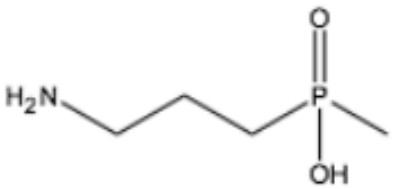


Figure 2.8: Drawings highlighting the chemical structure of the $GABA_B$ ligands used. Images created with ChemDraw Direct version 1.5.1.190.

During the project, drug delivery was executed in two ways: for 24 hours prior to the behavioural assay or for 24 hours between the second and third ethanol exposure.

2.6. Molecular Assays

In order validate the wild type and mutant fly lines for the functionality or not, reverse transcriptase polymerase chain reactions (RT-PCR) were attempted to assess the presence of a gene expression.

2.6.1. Primer Design

Primers were designed for the $GABA_B$ receptor 1 subunit and $GABA_B$ receptor 2 subunit. Primers were designed and purchased from Eurofins Genomics (Germany).

The $GABA_B$ R1 primer pair was derived by first obtaining the insertion point of the transposon. The sequence of the exon containing the insertion and locating the next exon were identified and the primer was designed on the two flanking exons. The intron was deemed to be 6572 bp (Table 2.3).

The $GABA_B$ R2 primer pair was obtained by again locating the insertion point of the transposon. The primer was designed on the exons flanking the insertion (Table 2.3).

A β Actin housekeeping gene primer was used as a known control. The primer was previously designed by a group member and was not designed specifically for this project.

Table 2.3: A table to portray the properties of the $GABA_B$ receptor subunit primers and β Actin control primers.

Primer ID	Sequence (5' to 3')	Product Length (bp)	Tm (ºC)
$GABA_B$ Receptor 1 Subunit **Forward**	TCGAACCTCATTTGCTCAGCG (21)	921	59.8
$GABA_B$ Receptor 1 Subunit **Reverse**	ATTTCAACGCCAGCCTCCAT (20)		57.3
$GABA_B$ Receptor 2 Subunit **Forward**	AACCAGTCAAATGGGTGGGG (20)	426	59.4
$GABA_B$ Receptor 2 Subunit **Reverse**	ACGTCTTCGAGAACATGGCT (20)		57.3
β Actin Control **Forward**	GCGTCGGTCAATTCAATCTT (20)	138	55
β Actin Control **Reverse**	AAGCTGCAACCTCTTCGTCA (20)		55

2.6.2. RNA Extraction

Ten *Drosophila* heads (5 male and 5 female) were dissected from each fly line and immediately snap frozen in liquid nitrogen for 5-10 minutes and was placed into RNA Later (Qiagen, USA) to both stabilised the RNA and to prevent RNA degradation. The heads of each fly line were then stored at -80ºC until the extraction. The RNA extraction was carried out using an RNeasy Mini Kit (Qiagen, USA) according to the manufactures instructions. The eluted RNA sample was then stored at -80ºC.

2.6.3. RT-PCR

The RT-PCR was executed using a reverse transcriptase kit (New England Biolabs, USA). The kit was used to synthesise cDNA as per the manufacturer's instructions. A PCR kit was then used (New England Biolabs, USA) following the manufacturers guidelines once more.

RT-PCR was performed to test for the presence of $GABA_B$ receptor subunit gene expression in the heads of wild type and mutant flies. The primers used for amplifying the PCR products of interest are listed in table 2.3.

For the cDNA synthesis, 20µl of RNA was used with a 10µl master mix containing a buffer, random primers, oligo-DT primers and dNTPs and reverse transcriptase; this reaction was carried out at 45°C for 20 min. The PCR reaction was carried out using 13µl of template cDNA from the previous reverse transcriptase reaction, 2.5µl of standard taq reaction buffer, 0.5µl of 10mM dNTP's, 1µl of forward/reverse primer mix (100µM), 0.125µl of taq DNA polymerase and 12.875µl of nuclease free water. The PCR was conducted within a thermocycler (Bio-Rad, USA) with a preheated block lid at 105ºC. The thermocycler was programmed as follows: initial denaturation at 95ºc for 2 minutes followed by 30 cycles of 10 seconds of denaturation at 95ºC, an annealing/extension at 56-60ºC for 30-60 seconds and a final extension for 5 minutes at 60ºC. Products were analysed on a 1% (w/v) agarose gel supplemented with Sybr Safe DNA gel stain (ThermoFisher Scientific, UK).

Chapter 3: Results

3.1. Ethanol Toxicity

To initiate the project, it was deemed important to establish what concentration of ethanol the *Drosophila* could consume before ethanol toxicity lead to high mortality. Many researchers such as Greenspan (2004), Ja *et al.* (2007), Stocker *et al.* (2008), Devineni and Heberlein (2009), Wong *et al.* (2009), Devineni and Heberlein (2012), Pohl *et al.* (2012), Peru *et al.* (2014) use concentrations within the range of 12-25% ethanol when administering ethanol in food. In order to investigate the validity of the reported data and to set standards for my experimentations, ethanol toxicity tests were executed by exposing and housing flies on standard dry mix food containing ethanol concentrations between 0% and 25% for 72 hours. 72 hours was decided as this was the maximum duration of time that the flies would ever be exposed to the ethanol for (Figure 3.1).

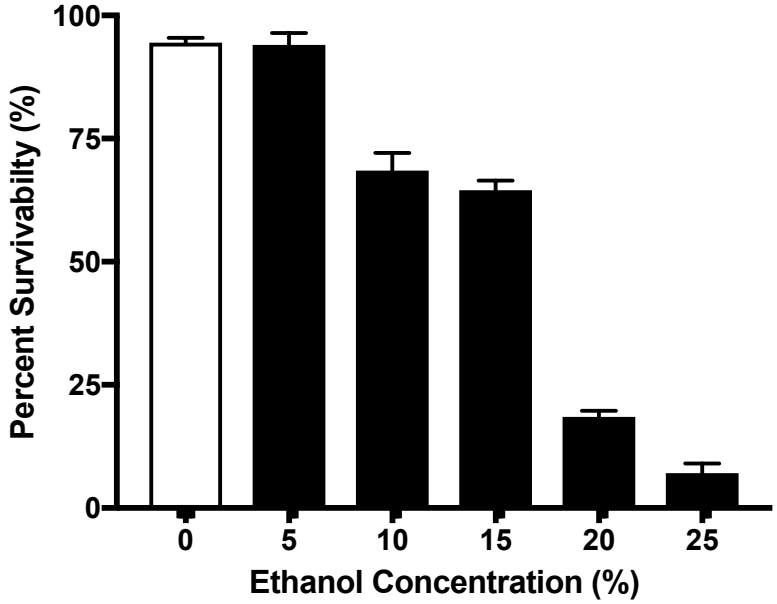


Figure 3.1: Percentage Survivability of *Drosophila* at various ethanol containing food concentrations.

10 males and 10 females aged 0-5 days were placed into separate vials with 0%, 5%, 10%, 15%, 20% or 25% ethanol containing dry mix solid foods for 72 hours. Vials were housed at 25°C. Number of mortalities was recorded per vial and calculated as a percentage. Values are ± SEM with n= 2 vials per sex (40 flies total).

The results from the toxicity testing indicate that the 20% and 25% ethanol containing foods resulted in greater than 75% mortality. Consequently, 10% and 15% ethanol containing foods resulted in around 30% mortality which was deemed acceptable. The food containing 5% ethanol resulted in almost no

mortality but from other studies within the lab, it is known that 5% ethanol induces very small behavioural changes. It was therefore decided that for all preference consumption experimentations, 15% would be the best concentration to use. The ability of the flies to survive at the lower concentrations of ethanol without mortality is consistent with their natural behaviour of seeking fermenting (ethanol containing) food for egg laying as previously stated by Pohl *et al.* (2012).

3.2. Tolerance Development in Wild Type and $GABA_B$ Mutant *Drosophila*

In Wild Type flies, repeated exposure to ethanol leads to a reduced sensitivity (tolerance) to its effect. To verify this phenomenon, tolerance was measured in wild type flies and compared to that of $GABA_B$ mutant flies (Figure 3.2). The mutants are putative functional knock outs (KO) for $GABA_B$ receptor subunits 1 ($GABA_B$ R1 KO) and 2 ($GABA_B$ R2 KO).

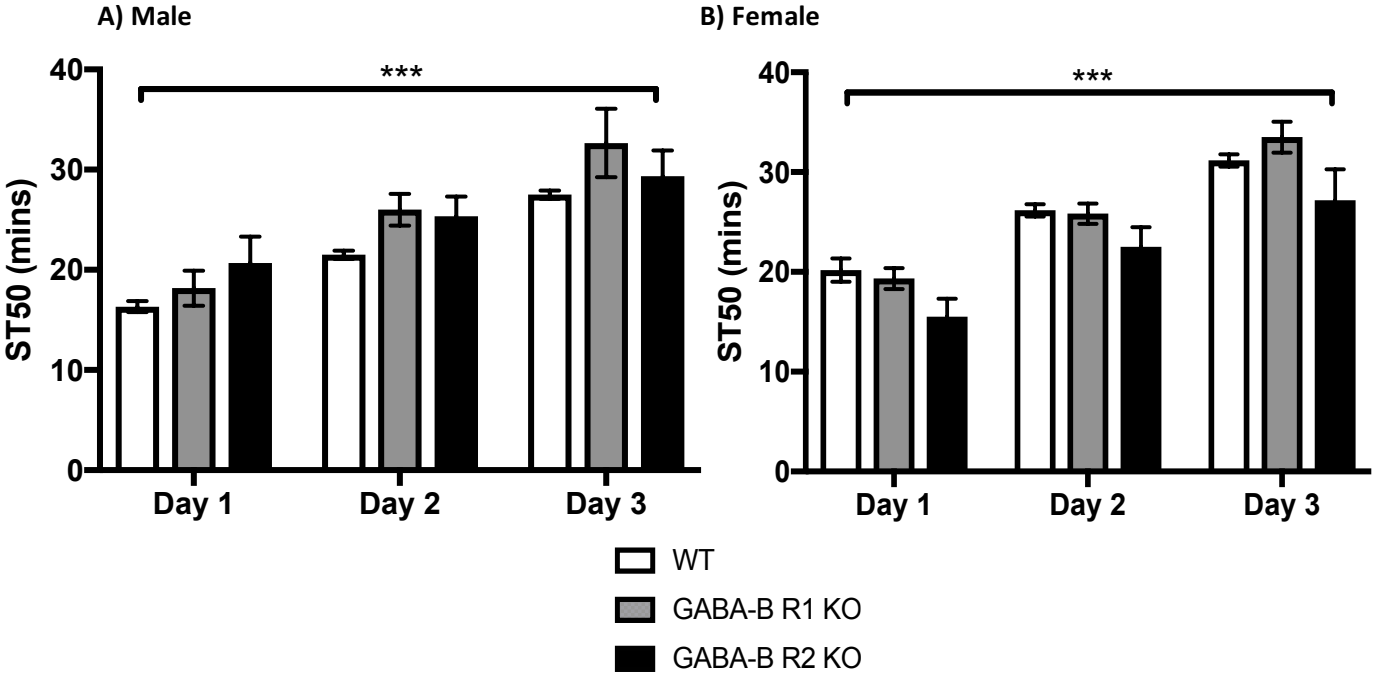


Figure 3.2: Tolerance development in three *Drosophila* lines for both A) Males, and B) Females.

*Tolerance of wild type, $GABA_B$ R1 KO and $GABA_B$ R2 KO male (A) and female (B) Drosophila. In each group 10 male/female flies were exposed to 500µL of 100% EtOH until ST50 was recorded. Values are mean ± SEM with n= 6 vial of 10 flies. A two-way ANOVA with Bonferroni post-hoc testing was performed with ***P<0.001 tolerance development for each individual fly line over the three days.*

Figure 3.2 demonstrates that time taken by half of the flies in the vial to become sedated increased in the wild type, $GABA_B$ R1 KO and $GABA_B$ R2 KO male and

female *Drosophila* strains indicating that they are all capable of developing tolerance to ethanol in a similar way. These findings strengthen and reinforce previously published works that the *Drosophila melanogaster* is a suitable model to study the development of tolerance to alcohol as an addiction like behaviour (Scholz *et al.* 2000, Atkinson 2009, Maples and Rothenfluh 2011). The fact that the $GABA_B$ mutants do not appear to behave differently from wild type does not necessarily invalidate their use, as discussed later.

3.3. $GABA_B$ Receptor Antagonist/Agonist Toxicity

There is no sufficient published data on what would be an effective and safe dose of CGP 54626 ($GABA_B$ receptor antagonist) or SKF 97541 ($GABA_B$ receptor agonist) in *Drosophila*, therefore a toxicity test was carried out. At all concentrations used, there were no 'abnormal' mortality patterns and it was decided to use the drugs at a concentration of 500nM, 250nM and 50nM (Toxicity data not shown).

3.4. The Effect of $GABA_B$ Receptor Drugs on the Development of Tolerance

Both $GABA_B$ receptor agonists and antagonists were administered for 24 hours prior to day 1 of the tolerance assay.

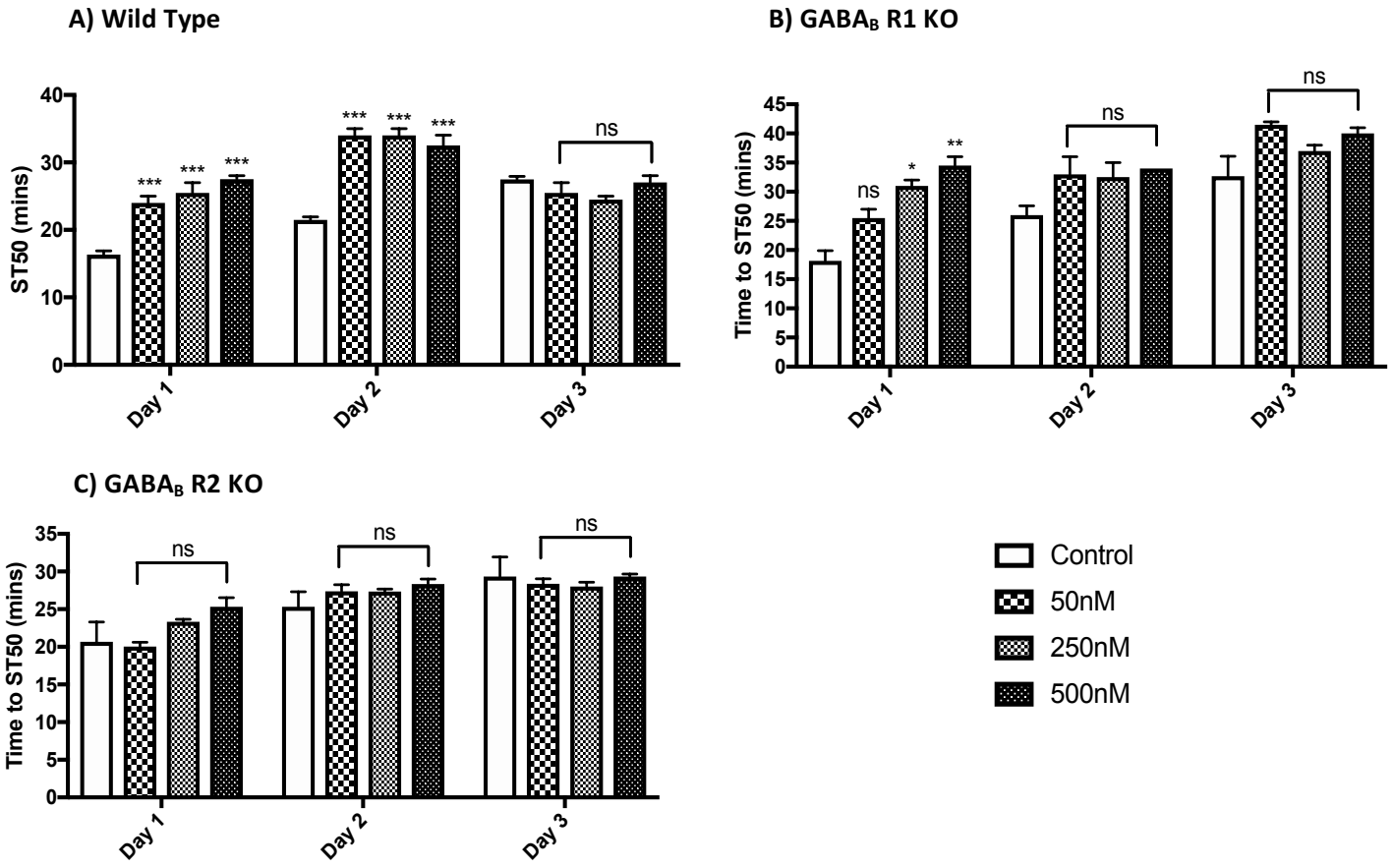


Figure 3.3: Effect of $GABA_B$ receptor antagonist CGP 54626 administered for 24 hours prior to the 3 day tolerance assay.

*Tolerance of Wild Type **(A)**, $GABA_B$ R1 KO **(B)** and $GABA_B$ R2 KO **(C)** male Drosophila. $GABA_B$ receptor antagonist CGP 54626 was administered for 24 hours prior to day 1 of the tolerance assay. In each vial, 10 male flies aged 0-5 days old were exposed to 500µL 100% EtOH until ST50 was recorded. Values are mean ± SEM with n= 3 vials of 10 flies (30 flies total) and control n= 6 vials of 10 flies (60 flies total). A two-way ANOVA with Bonferroni post-hoc testing was performed with * P<0.05, **P<0.01, ***P<0.001 ns= P>0.05 when compared to the relevant control for the corresponding day.*

The antagonist CGP 54626 was able to enhance and increase the tolerance development in the wild type and $GABA_B$ R1 KO when compared to the control (Figure 3.3) in day 1 and day 2 of the assay but not in day 3. It is worth noting that within these experiments the drug was only administered before the assay and not during the three day assay. Conversely from the effect on $GABA_B$ receptor R1 KO, CGP 54626 had no significant effect on $GABA_B$ R2 KO (Compared to Day 1 R2 KO control: 50nM P=>0.9999 250nM P=0.9999, 500nM P=0.7018).

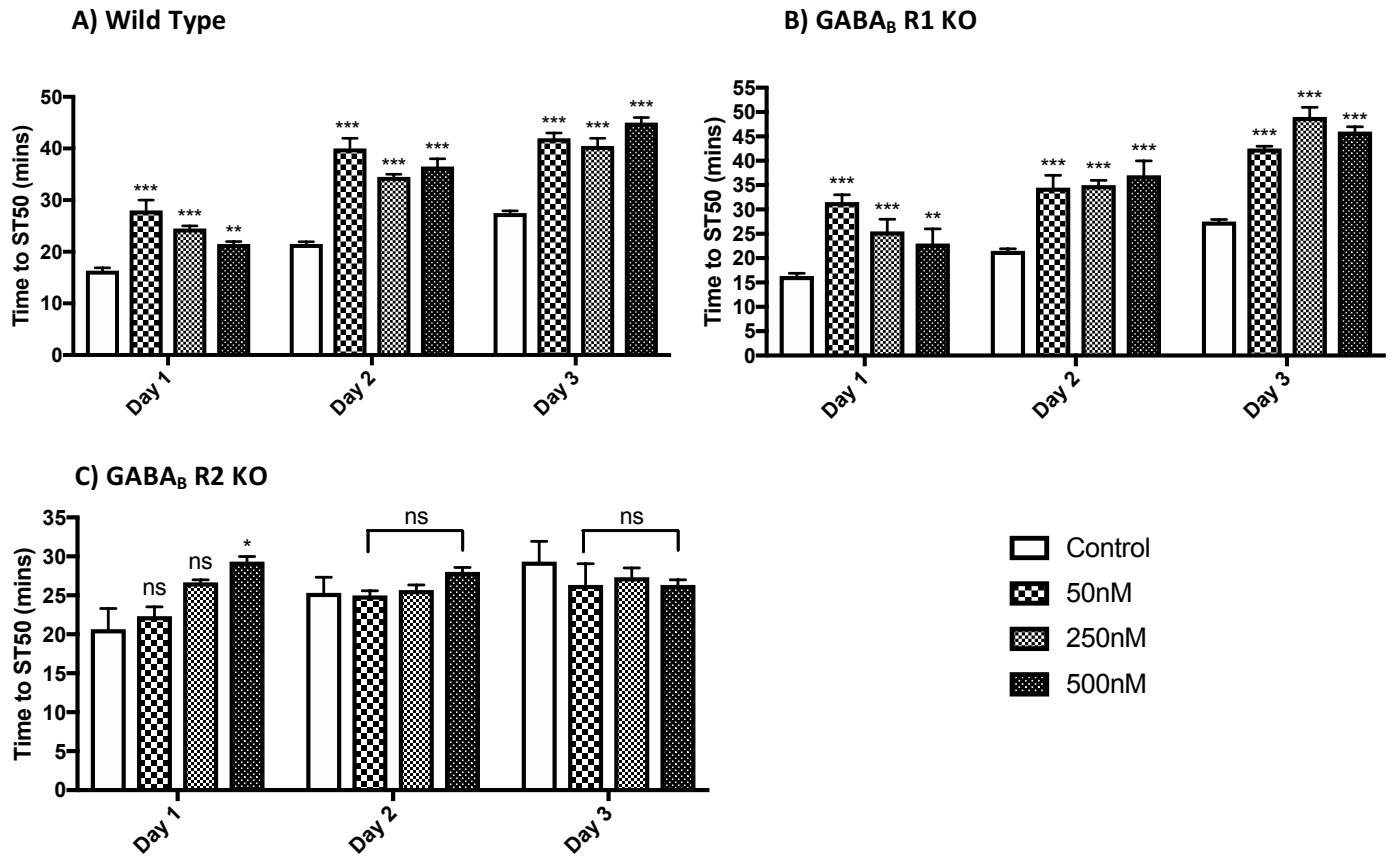


Figure 3.4: Effect of $GABA_B$ receptor agonist SKF 97451 administered for 24 hours prior to the 3 day tolerance assay.

*Tolerance of Wild Type **(A)**, $GABA_B$ R1 KO **(B)** and $GABA_B$ R2 KO **(C)** male Drosophila. $GABA_B$ receptor agonist SKF 97541 was administered for 24 hours prior to day 1 of the tolerance assay. In each vial, 10 male flies aged 0-5 days old were exposed to 500µL 100% EtOH until ST50 was recorded. Values are mean ± SEM with n= 3 vials of 10 flies (30 flies total) and control n= 6 vials of 10 flies (60 flies total). A two-way ANOVA with Bonferroni post-hoc testing was performed with * P<0.05, **P<0.01, ***P<0.001 when compared to the relevant control for the corresponding day. ns= P>0.05 indicating no statistical significance when compared to the relevant control for the corresponding day.*

The effect of the agonist SKF 97541 was also tested on wild type and mutant flies (Figure 3.4). The agonist and the antagonist acted in a similar way, and both were able to enhance and increase the tolerance development in the wild type and $GABA_B$ R1 KO when compared to the control (Figure 3.4). The $GABA_B$ R2 KO was again, unable to develop a significant increase in tolerance (Compared to Day 1 R2 KO control: 50nM P=>0.9999 250nM P=0.3350, 500nM P=0.0434).

When compared to the antagonist, certain comparisons are evident between the agonist and antagonist drugs delivered prior to the tolerance assay. Both drugs appear to lose efficacy after day 2/3; this is suspected to be as a result of the

drug being metabolised. In order to maintain effect, the drug would need to be administered on a daily basis Interestingly, the agonist and the antagonist appear to be working in a similar way. Based on published literature, it would be expected that the agonist and antagonist would work in opposite ways. A possible reason for why this is not occurring is that the agonist is operating as a partial agonist and is reducing the effect of the endogenous GABA just like the antagonist.

3.5. Reversing the Development of Tolerance with $GABA_B$ Receptor Drugs

Using a modified protocol to the experiments described in the previous section, tolerance was developed in the *Drosophila* for two days and after the second day, the $GABA_B$ receptor antagonist and agonist were administered for 24 hours before day 3 of the tolerance experiment whilst the control vials were administered a standard dry food placebo. This delivery was used to challenge an established ethanol tolerance via administration of GABA-B receptor agonists and antagonists.

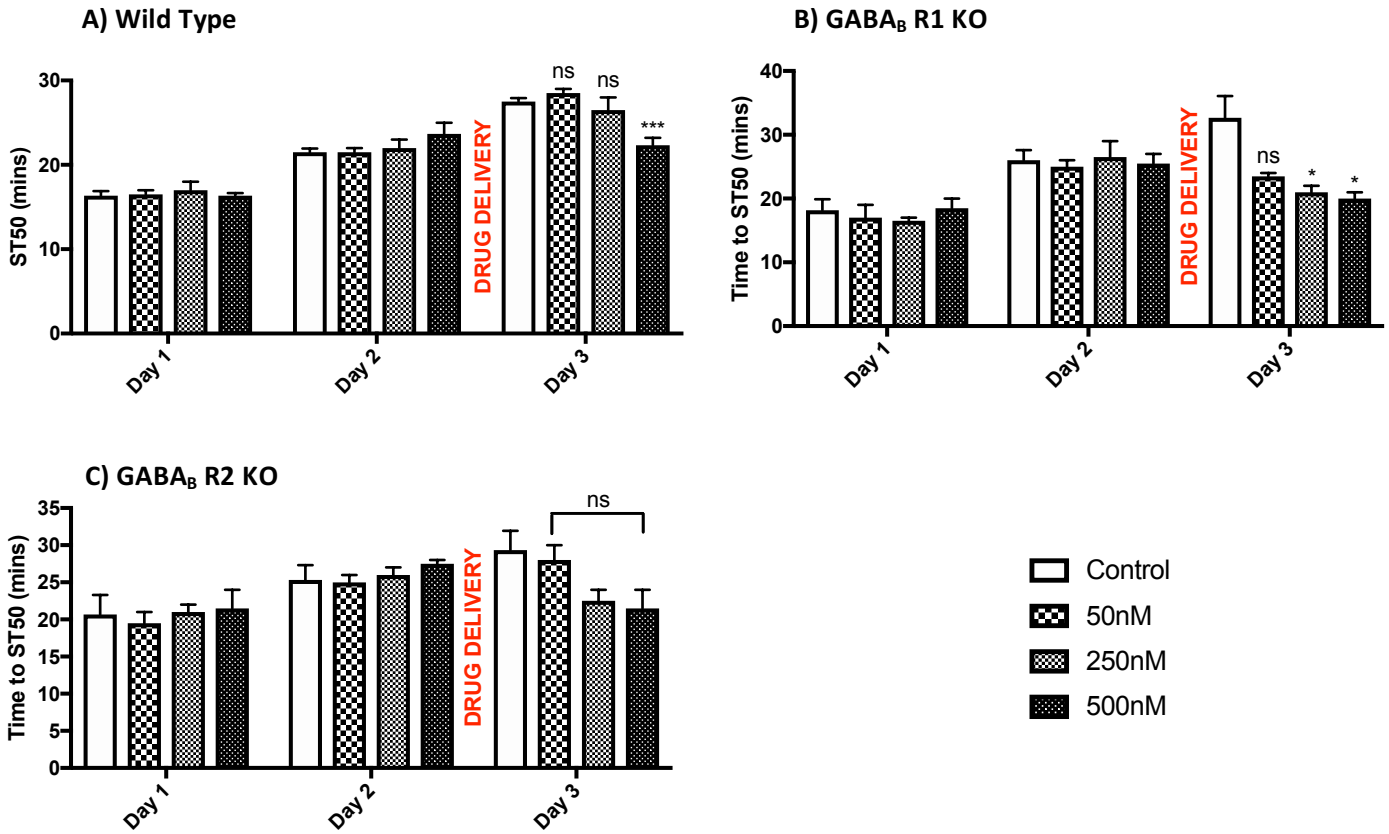


Figure 3.5: The effect of $GABA_B$ receptor antagonist CGP 54626 on flies pre-exposed to ethanol for 2 days in the tolerance assay.

*$GABA_B$ receptor antagonist CGP 54626 was administered to Wild Type **(A)**, $GABA_B$ R1 KO **(B)** and $GABA_B$ R2 KO **(C)** male Drosophila. for 24 hours after day 2 of the tolerance assay. In each vial, 10 male flies aged 0-5 days old were exposed to 500µL 100% EtOH until ST50*

*was recorded. Values are mean ± SEM with n= 3 vials of 10 flies (30 flies total) and control n= 6 vials of 10 flies (60 flies total). A two-way ANOVA with Bonferroni post-hoc testing was performed with * P<0.05, ***P<0.001 Ns (not significant) = P>0.05 when compared to the relevant control for the corresponding day.*

The results shown in figure 3.5, indicate that in wild type flies, the CGP 54626 antagonist when administered after day 2 does not increase tolerance as it had been observed when administered before starting the tolerance assay (figure 3.3) but at the highest dose actually decreases ST50. This reversal of the tolerance effect is even more evident in the $GABA_B$ R1 KO but is not statistically significant in the $GABA_B$ R2 KO flies.

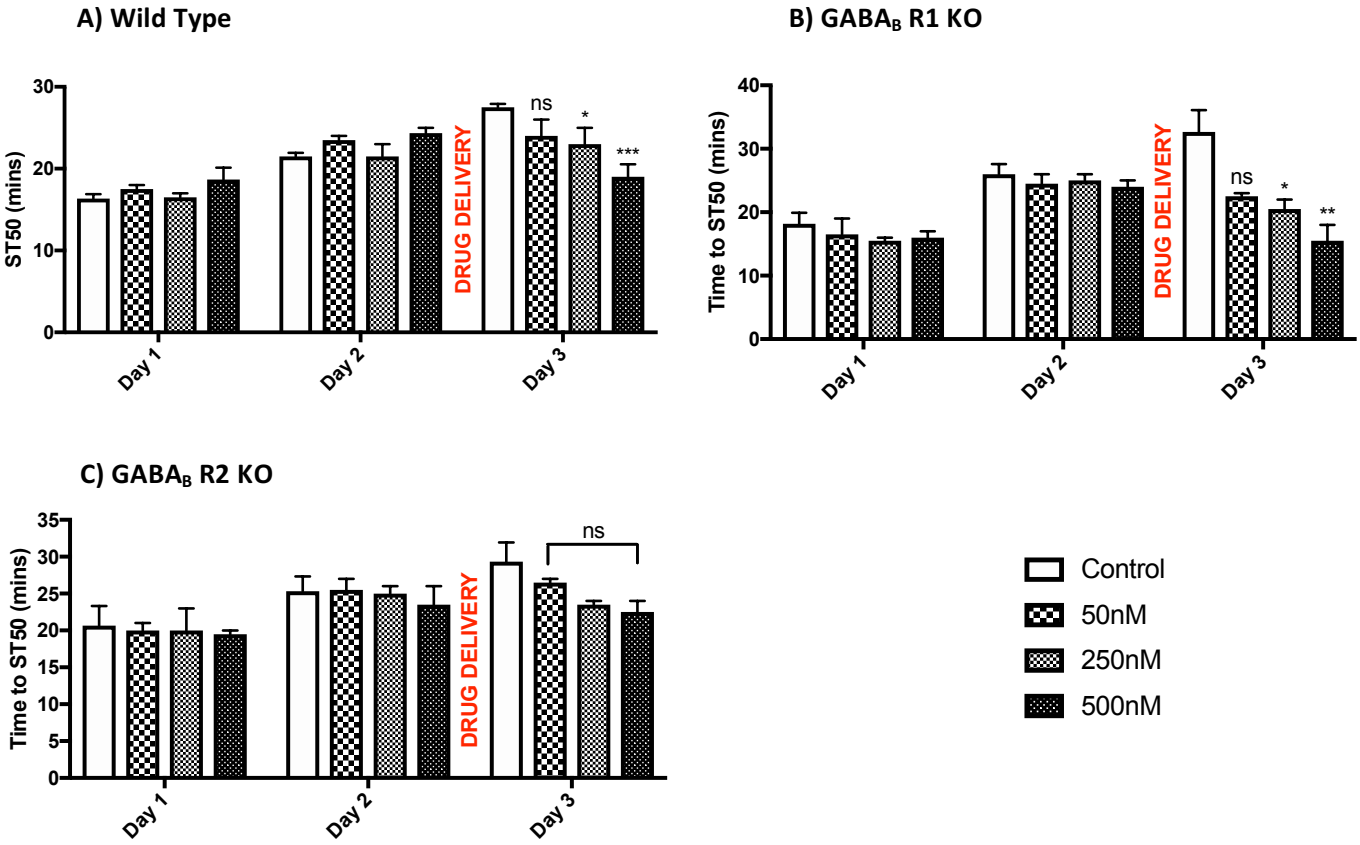


Figure 3.6: The effect of $GABA_B$ receptor agonist SKF 97451 on flies pre-exposed to ethanol for 2 days in the tolerance assay.

$GABA_B$ receptor agonist SKF 97541 was administered for 24 hours to *Wild Type **(A)**, $GABA_B$ R1 KO **(B)** and $GABA_B$ R2 KO **(C)** male Drosophila. after day 2 of the tolerance assay. In each vial, 10 male flies aged 0-5 days old were exposed to 500µL 100% EtOH until ST50 was recorded. Values are mean ± SEM with n= 3 vials of 10 flies (30 flies total) and control n= 6 vials of 10 flies (60 flies total). A two-way ANOVA with Bonferroni post-hoc testing was performed with *P<0.05, ***P<0.001, ns (not significant) = P>0.05 when compared to the relevant control for the corresponding day.*

The $GABA_B$ agonist used after day 2 of tolerance development appears to act in the same way that the administered antagonist does on tolerance for the wild type and $GABA_B$ R1 KO mutant after the drug has been administered (Figure 3.5/ 3.6).

Remaining consistent to all previous results described in section 3.3 and section 3.4, the $GABA_B$ R2 KO mutant does not respond to the drug delivered and shows no changes to tolerance when challenged after two days of tolerance development (Figure 3.6). Whilst the graph appears to show a decrease in tolerance development, the results indicate no statistical significance (Compared to Day 3 R2 KO control: 50nM P=>0.9999, 250nM P=0.9442, 500nM P= 0.6012).

3.6. Preference Development in Wild Type and $GABA_B$ Mutant *Drosophila*

In Wild Type flies, repeated exposures to liquid foods with or without ethanol over a period of time leads to an increased preference to one food or another. To validate this process, *Drosophila* were allowed to consume the liquid food or ethanol containing food presented to them in separate capillary tubes and the preference index for the two types of food was calculated based upon the consumption rates of each food. The preference index of $GABA_B$ mutant flies were compared to that of Wild Type flies (Figure 3.7).

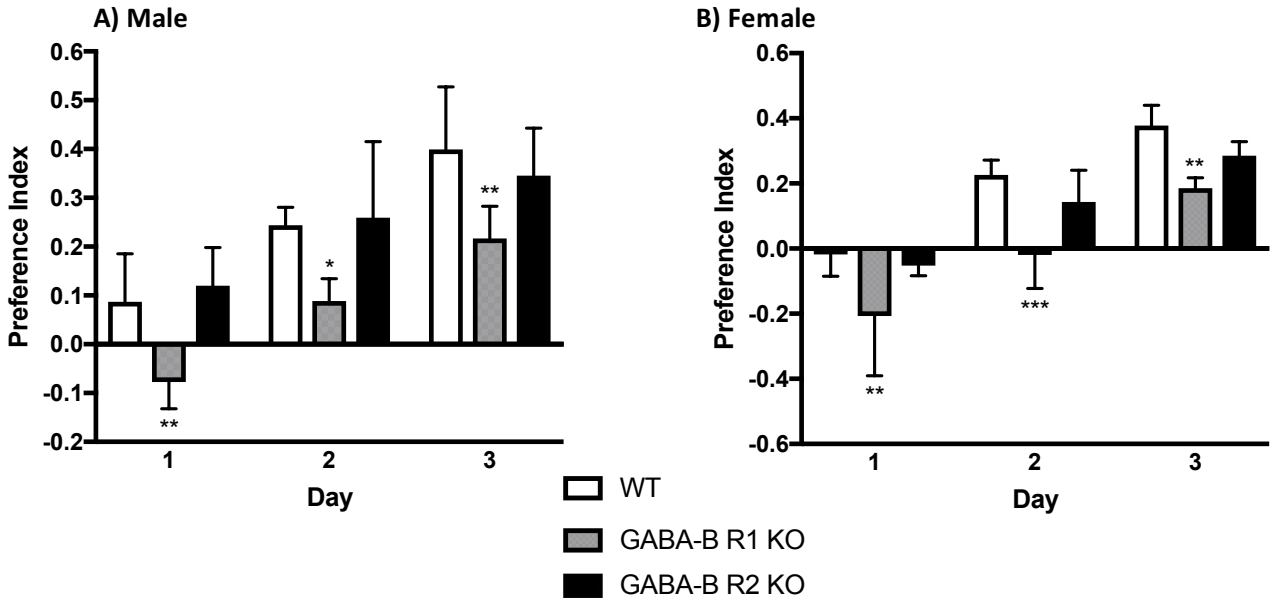


Figure 3.7: Preference development in males and females of three *Drosophila* lines

*The preference assays were repeated for 3 consecutive days for wild type, $GABA_B$ R1 KO and $GABA_B$ R2 KO male **(A)** and female **(B)** Drosophila aged 0-5 days. Drosophila were housed on standard dry mix food in between assays. Preference Index (consumption of ethanol containing food against non-ethanol containing food) is reported as mean ± SEM with n= 6 vials of 10 flies (60 flies total). Data was analysed by two-way ANOVA with Bonferroni post-tests. * P<0.05, ** P<0.01, *** P<0.001 when compared to the WT control of the same day.*

Figure 3.7 demonstrates that preference to 15% ethanol liquid food was developed by the wild type, $GABA_B$ R1 KO and $GABA_B$ R2 KO male and female *Drosophila* over three days and strengthens and reinforces previously published works that the *Drosophila melanogaster* is a suitable model to study preference as an addiction like behaviour to alcohol (Ja *et al.* 2007, Devineni and Heberlein 2009, Pohl *et al.* 2012, Peru *et al.* 2014).

It can be said, that by looking at the preference development over days for all fly lines, that preference for ethanol containing food vs non-ethanol containing liquid food occurs much quicker in the wild type and the $GABA_B$ R2 KO mutant than the $GABA_B$ R1 KO mutant. This is not coincidental as both the male and female groups act in the same way and are statistically different from the wild type species with no $GABA_B$ receptor transgenic mutation. (Figure 3.7).

3.7. The Effect of $GABA_B$ Receptor Drugs on Development of Preference

As tolerance development to alcohol was affected by $GABA_B$ drugs (section 3.3), it was questioned whether preference could be too. The same $GABA_B$ antagonist CGP 54626 used for tolerance interference and as described in section 2.5 was used during this assay. The antagonist was administered to male *Drosophila* for 24 hours prior to day 1 of the preference assay (Figure 3.8). Only males were used due to project time constraints.

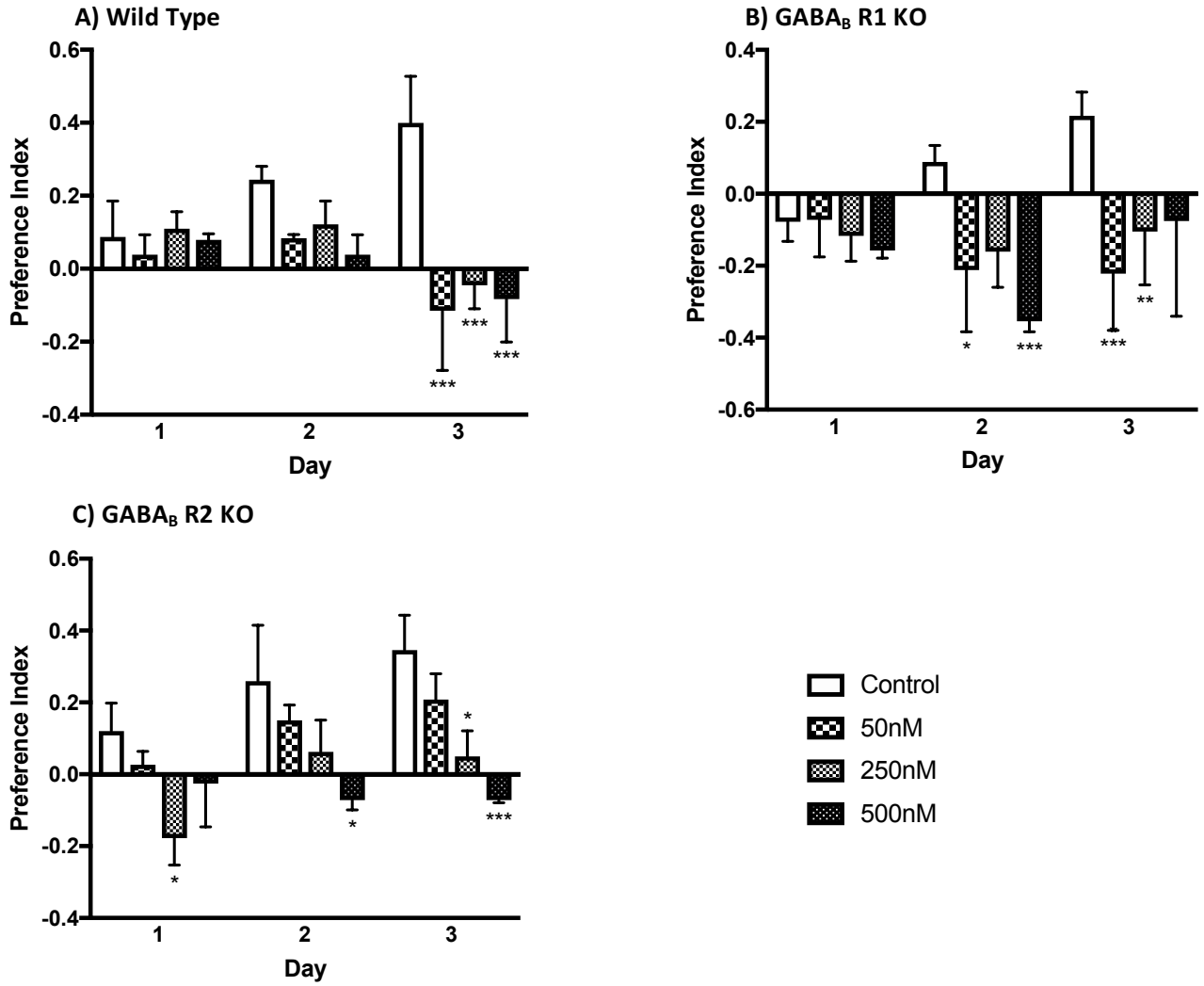


Figure 3.8: Effect of $GABA_B$ receptor antagonist CGP 54626 administered for 24 hours prior to 3 day preference assay.

*$GABA_B$ receptor antagonist CGP 54626 was administered for 24 hours to **(A)** Wild Type, **(B)** $GABA_B$ R1 KO and **(C)** $GABA_B$ R2 KO male Drosophila aged 0-5 days. The preference assays were repeated for 3 consecutive days with no further exposure to the antagonist. Drosophila were housed on standard dry mix food in between assays. Preference Index (consumption of ethanol containing food against non-ethanol containing food) is reported*

*as mean ± SEM with n= 3 vials of 10 flies (30 flies total) and control n= 6 vials of 10 flies (60 flies total). Data was analysed by two-way ANOVA with Bonferroni post-tests. * P<0.05, ** P<0.01, *** P<0.001 when compared to the WT control of the same day.*

The consumption of the antagonist CGP 54626, produced a developmental response in the different fly lines (Figure 3.8), whereas the agonist SKF 97541 (Figure 3.9) consumed by the *Drosophila* prior to day 1 of the initial preference assay appear to have a delayed mechanism of action. For all *Drosophila* lines at all doses and drugs administered for the preference assay (with the exception of $GABA_B$ R1 KO at 500nM agonist and $GABA_B$ R2 KO at 250nM antagonist), there is no significant impact and no statistically significant antagonist or agonist activity on preference from the drug on day 1 of the preference assay.

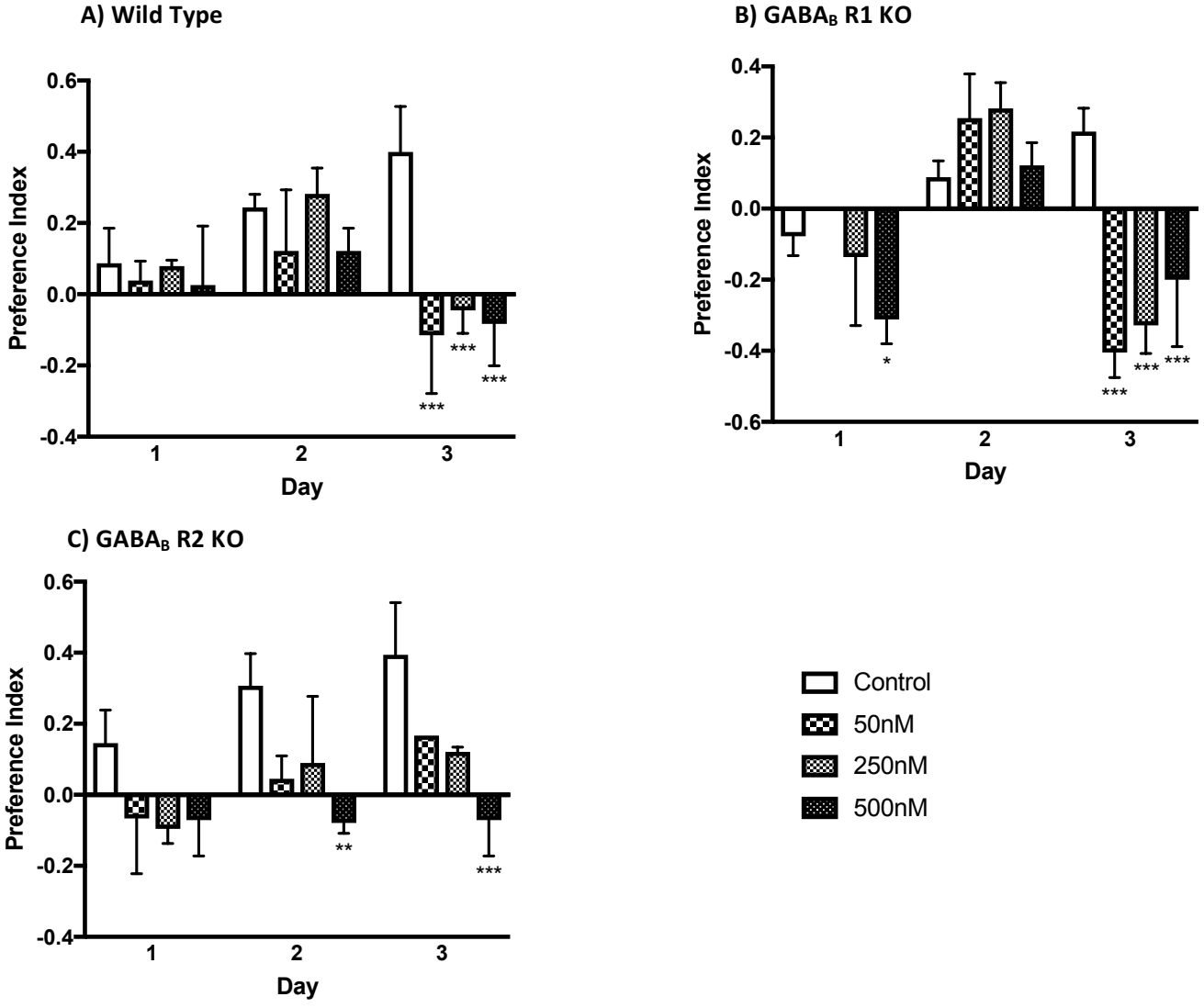


Figure 3.9: Effect of $GABA_B$ receptor agonist SKF 97451 administered for 24 hours prior to the 3 day preference assay.

*$GABA_B$ receptor agonist SKF 97541 was administered for 24 hours to **(A)** Wild Type, **(B)** $GABA_B$ R1 KO and **(C)** $GABA_B$ R2 KO male Drosophila aged 0-5 days. The preference assays were repeated for 3 consecutive days with no further exposure to the agonist. Drosophila were housed on standard dry mix food in between assays. Preference Index (consumption of ethanol containing food against non-ethanol containing food) is reported as mean ± SEM with n= 3 vials of 10 flies (30 flies total) and control n= 6 vials of 10 flies (60 flies total). Data was analysed by two-way ANOVA with Bonferroni post-tests. ** P<0.01, *** P<0.001 when compared to the WT control of the same day.*

3.8. Conditional Preference Assay Validity/Controls

In order to confirm and validate the *Drosophila* as a model for observing unbiased preference development to alcohol, the conditional preference assay was carried out where flies are pre-trained to associate alcohol vapours with one of two neutral odour and then are observed making choice for the odours in absence of any alcohol vapours as described in section 2.4.3.

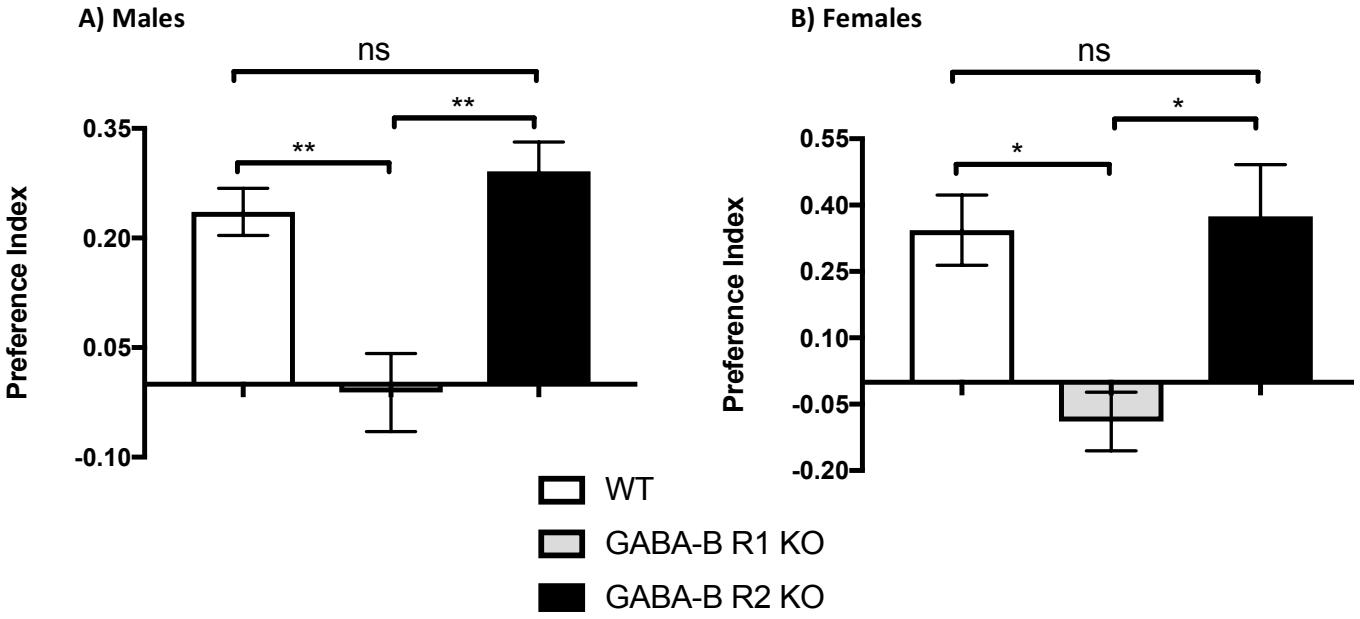


Figure 3.10: Conditional preference to ethanol in males and females from three *Drosophila* lines.

*Conditional preference was measured in Wild Type, $GABA_B$ R1 KO and $GABA_B$ R2 KO male **(A)** and female **(B)** Drosophila aged 0-5 days. Flies were kept in vials at 25°C and at room temperature whilst training. Flies were trained for a total of 30 minutes on each odour (Odour A + EtOH & Odour B + H_2O) for 10-minute training sessions separated with a 10 minute rest. MCH was EtOH associated odour and OCT was H_2O associated odour. In the conditional preference test (24 hours after the last training session) flies were presented with odours via opposite ends of the 'Y Maze' test apparatus at a rate of 1.71 litres per minute Preference Index (movement to ethanol associated odour against movement to H_2O associated odour within 3 min) is reported as mean ± SEM with n= 6 vials of 20 flies (120 flies total). Data was analysed by one-way ANOVA with Bonferroni*

*post-test. * P<0.05 ** P<0.01 and ns (not significant) = P>0.05 indicating no statistical significance when differences compared between indicated species.*

By looking at the data presented in Figure 3.10, it can be concluded that both male and female wild type and $GABA_B$ R2 KO *Drosophila* lines, when given an unbiased movement choice, prefer to move to the ethanol associated odour and show a strong positive preference after odour association training. The $GABA_B$ R1 KO however for both sexes show a negative preference to the ethanol associated odour.

This assay is valuable in reinforcing the preference assay behavioural results (section 3.6). This conditional preference assay allows the *Drosophila* a free unbiased choice to show preference to an ethanol associated odour or a water associated odour. The main differences in the design of the two preference based assays is that within the conditional preference, the *Drosophila* never come into direct contact with the ethanol, indicating consumed and olfactory preference to ethanol and ethanol is not present during the assay (only during training).

3.9. RT-PCR

Despite several attempts at validating the *Drosophila* wild type and mutants used within this project no products were obtained from the RT-PCR amplifications. Unfortunately, these validation experiments were executed at the end of the project and there was not enough time to trouble shoot the ineffectiveness of the assay other than varying the annealing temperature (56-60°C) and the extension time (30-60 sec). The most likely explanations are the mRNA preparations were not of good enough quality or one of the enzymes used was not functioning as expected.

Chapter 4: Discussion

4.1. *Drosophila* as Model for Studying Addiction

Alcohol misuse is a problem worldwide and was responsible for 3.3 million deaths in the year 2015 (World Health Organistation [1] 2015). Alcohol is thus responsible for more global deaths than chronic obstructive pulmonary disease deaths (3.1 million), trachea, bronchus and lung cancers (1.7 million) and diabetes mellitus (1.6 millions deaths) (World Health Organistation [2] 2017).

It is because of the number of alcohol related deaths and the socio-economic impact of alcohol addiction that an intervention is necessary and should be a focus of researchers. It is in this context that this project was aimed at understanding the role of the $GABA_B$ receptor in preference and tolerance to alcohol to lay foundations for future work and to assess if the receptor could be used as a possible pharmacological target to address addiction like behaviours.

One of the main objectives of the project was to investigate further the role and suitability of the *Drosophila* melanogaster as a model for studying alcohol induced behaviours and their underlying molecular mechanisms. The results obtained in this study confirm that following ethanol exposures over three consecutive days, the wild type *Drosophila* can develop a significant increase in their ST50 values which can be identified as tolerance (Figure 3.2). In the second assay employed in this project, where the wild type *Drosophila* have free choice and access to both ethanol containing food and non-ethanol containing food, it is evident from figure 3.7 that the wild type *Drosophila* consume more ethanol containing food over consecutive days, resulting in an increased preference index. Thus, a stronger preference to ethanol is established over time with repeated exposures to ethanol. In the third assay employed in this project, when flies were exposed to two different neutral olfaction odours at separate times alongside ethanol or H_2O, the wild type *Drosophila* when placed into a Y maze testing apparatus made a free choice to move towards the ethanol associated odour (Figure 3.9). This reinforces the results from the primary preference assay within figure 3.7, that a positive preference to ethanol can be established and demonstrated as an addiction like behaviour in wild type *Drosophila*.

The ability to develop and maintain these behaviours further confirms the works as described by Scholz *et al.* (2000), Ja *et al.* (2007), Devineni and Heberlein

(2009), Maples and Rothenfluh (2011), Kaun *et al.* (2012), Pohl *et al.* (2012), Simonnet *et al.* (2014), Umukoro (2015), Koyyada (2016) and confirms the fruit fly as being a suitable model to study the mechanisms and effects of alcohol addiction.

4.2. $GABA_B$ Receptor Ligands

Initial evidence of addiction like behaviours being suppressed by $GABA_B$ receptor agonists (Hwa et al. 2013), and the report that silencing the $GABA_B$ receptor gene reduced the behaviour impairing effects of ethanol (Dzitoyeva et al. 2003), led to the selection of the $GABA_B$ receptor as the main focus of this study and, in particular, the role of this receptor in the development of tolerance and preference was targeted. The $GABA_B$ receptor antagonist selected was compound CGP 54626 and was selected as it is described as being a 'selective $GABA_B$ antagonist' (Tocris [1] 2016). The $GABA_B$ receptor agonist selected was compound SKF 97541 and was chosen based upon the description given from the supplier detailing 'Very potent $GABA_B$ agonist, at least ten times more active than baclofen' (Tocris [2] 2016). The supplier's statements that the drugs were potent and specific receptor ligands were of great appeal for this project. It is important to note that some $GABA_B$ drugs do not have an effect on the *Drosophila* such as the highly used $GABA_B$ receptor agonist Baclofen (Manev and Dzitoyeva 2010).

Being able to interfere with the development/maintenance of the addiction like behaviours that the *Drosophila* have demonstrated in section 4.1.1 was an area of interest in this project. By administering both drugs independently, it was of interest as to how the behaviours would change in the presence of ethanol. Drugs were either administered 24 hours prior to the first exposure to ethanol or administered for 24 hours after two days exposure to ethanol to investigate if the behaviour would change.

4.3. Efficacy of $GABA_B$ Receptor Drugs on the Development of Tolerance in Wild Type and Mutant *Drosophila*

The wild type and mutant *Drosophila* at all stages of this project developed tolerance addiction behaviours upon exposure to ethanol. When a $GABA_B$ receptor ligand was administered for 24 hours prior to the tolerance assay, there

was some changes in the development of tolerance and this was different for each fly line in the presence of the agonist or antagonist.

Administration of $GABA_B$ receptor antagonist to wild type *Drosophila* for 24 hours prior to the first ethanol exposure of the tolerance assay caused a significant increase of ST50 at all concentrations tested (50nM, 250nM and 500nM) This increase was sustained for day 1 and day 2 but not day 3 of the tolerance assay (Figure 3.3a). A possible explanation for this is that CGP 54626 is fully metabolised after 48 hours.

Mutant flies in which the $GABA_B$ receptor 1 subunit has been putatively knocked out by a genetic insertion ($GABA_B$ R1 KO) acts in a similar way to the wild type fly for the first day, but for days 2 and 3, there is an apparent decrease in the efficacy of the antagonist (Figure 3.3b). It must be noted however, that there was no significance at 50nM when compared to the control on day 1.

Mutant flies in which the $GABA_B$ receptor 2 subunit has been putatively knocked out by a genetic insertion ($GABA_B$ R2 KO) had no statistical difference for any day after being administered the antagonist CGP 54626 for 24 hours (Figure 3.3c). For day 1, visually there could be some difference for the 500nM concentration, but data analysis indicates no statistical significance (n=3 for treated and n=6 for control). More repeats of this fly line may give a greater indication and a true representation for the population and may in turn lead to significance. If this fly was to be a true knock out of the $GABA_B$ receptor 2 subunit, it indicates that the R2 subunit is needed for the increase of ST50 induced by CGP 54626.

The $GABA_B$ receptor agonist (SKF 97541) administration was also carried out for 24 hours prior to the first ethanol exposure of the tolerance assay, and interestingly, the agonist, like the antagonist, also significantly increased the ST50 of the wild type *Drosophila* (Figure 3.4a). The ST50 of agonist treated flies at all concentrations were significantly increased when compared to the control for that day. The effect remained for all three days of tolerance assay. It would be difficult to determine whether the drug is still present in the flies after three days. Therefore, it would be useful to continue the assay over a greater number of days

to investigate the duration of the drug effect. If the effect of the drug were to continue and be significant over more consecutive days, it would indicate that there has been some lasting changes in the *Drosophila* that had been exposed to the $GABA_B$ agonist. It would be of interest to determine the longevity of the drug but it would probably require extraction and a highly sensitive chromatography approach to detect the metabolism of the drug in the flies. It would be of even greater interest to determine if there are indeed any permanent behavioural changes caused by the drug exposure.

In these tolerance experiments, the agonist induced the same behavioural effect as an antagonist by increasing the ST50 of wild type *Drosophila*. When these conditions occur, a simple explanation is that the agonist is acting as a partial agonist rather than a full agonist. A partial agonist would be an agonist with lower efficacy than the endogenous agonist and thus by competing with the endogenous agonist, the partial agonist will have a similar effect as an antagonist. It can also be noted from the data that the lower concentration (50nM) of the agonist, has a statistically greater effect on increasing the ST50 value than the higher concentration (500nM) ($P<0.05$). There is no clear explanation for this observation, but it may suggest that the role of the $GABA_B$ receptor on the development of tolerance is more complex than first envisaged. One potential reasoning for this is that the drugs bind at two distinct sites; At low ligand concentrations, the high affinity binding site initiates ‘effector 1’ that is responsible for increasing the ST50/ sensitivity to ethanol and at higher ligand concentrations, the drug binds to a low affinity binding site that induces ‘effector 2’ and this is responsible for decreasing the ST50 and subsequently, the flies sensitivity to ethanol by altering the equilibrium of this mechanism (Figure 4.1).

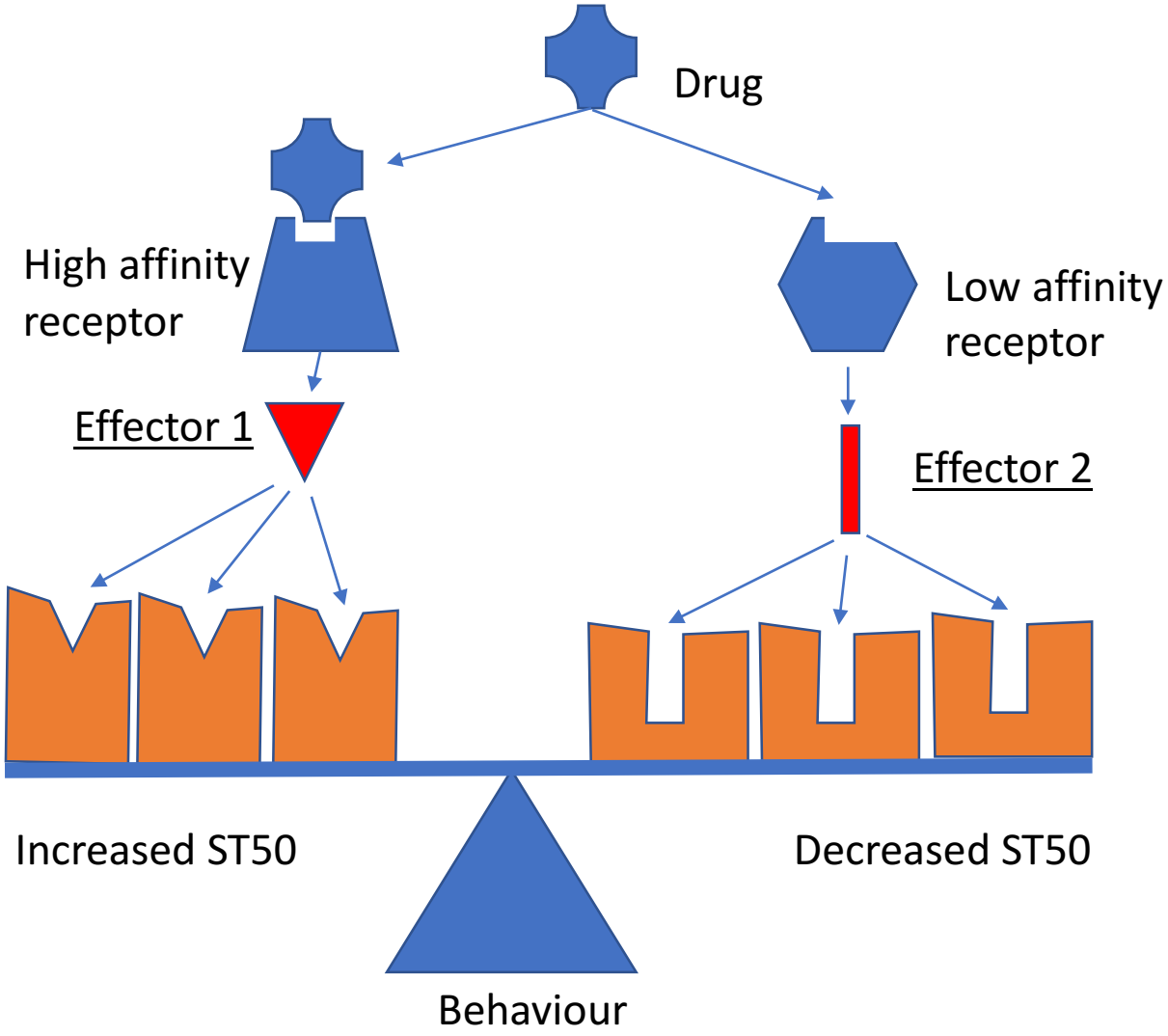


Figure 4.1: Concept of a high affinity/low affinity design for ligand concentration effects on tolerance behaviours.

At low ligand concentrations, the high affinity binding site initiates 'effector 1' that is responsible for increasing the ST50/ sensitivity to ethanol and at higher ligand concentrations, the drug binds to a low affinity binding site that induces 'effector 2' and this is responsible for decreasing the ST50 and subsequently, the flies sensitivity to ethanol.

4.4. Efficacy of $GABA_B$ Receptor Drugs on the Interference of Tolerance Development in Pre-Exposed Wild Type and Mutant *Drosophila*

In the experiments discussed in section 4.3, the $GABA_B$ receptor ligands were given before the ethanol exposures. Figures 3.5 and 3.6 portray a new set of experiments that were carried out whereby the drugs were administered for 24 hours after the second ethanol exposure. The rationale for this was to test whether the drugs were able to affect the development of tolerance after

tolerance development had already been initiated. There were some changes in the development of tolerance and this was different for each fly line in the presence of the agonist or antagonist.

For the wild type fly line, when the $GABA_B$ receptor antagonist CGP 54626 was administered for 24 hours after the second day tolerance assay, there was some change in the ST50 tolerance development (Figure 3.5a). At the higher concentration level (500nM), there was a significant ($P<0.001$) decrease in ST50 compared to the control on day 3. This result equalled the control of day 2 (before the administration of the antagonist) and therefore shows that the antagonist at this concentration is able to reverse tolerance development when administered after some tolerance development. The lower concentrations (50nM and 250nM) produced no statistically significant effects compared to the control and it can be said therefore that a higher concentration leads to lowering the ST50 trend. In future works, it would be interesting to see if a higher than 500nM concentration of the antagonist CGP 54626 could lower the ST50 further; potentially to the day 1 control tolerance level or potentially even lower.

The $GABA_B$ R1 KO mutant line, acts in a similar way to the wild type, with only the higher concentrations inducing an effect and showing a significant difference on tolerance ST50 after administration of the antagonist after day 2 of the tolerance assay (Figure 3.5b). However, the two fly lines differ in how much of an effect the drug can induce. The $GABA_B$ receptor ligand at the 250nM and 500nM were able to interfere with tolerance development and reduce the ST50 to between the tolerance controls for days 1 and 2. This was greater than the effect of the antagonist induced on the wild type *Drosophila.*

The $GABA_B$ R2 KO was not responsive to any concentration of the antagonist CGP 54626 (Figure 3.5c). This fly line, with only an active $GABA_B$ receptor 1 subunit, has similarities with the wild type *Drosophila* line. With this fly line and the wild type showing little to no tolerance development changes in the presence of an antagonist, this highlights and presents evidence that the R2 subunit is involved with the reversal of tolerance as the $GABA_B$ R1 KO (Figure 3.5b).

When the $GABA_B$ receptor agonist SKF 97541 was administered in the same way as the antagonist after day 2 for 24 hours, the results for the wild type *Drosophila* and mutant $GABA_B$ R1 and R2 KO lines, were nearly identical to the those obtained from the antagonist results (Figure 3.6). The higher concentrations of the drug were the only ones responsible for inducing an effect and overcoming the presence and sensitivity to the ethanol. The $GABA_B$ R1 KO acted the same as the wild type fly line as it has done in previous experiments, and as previously seen, the R2 KO was seen to still show no change when administered a $GABA_B$ receptor ligand, indicating the importance of the receptor 2 subunit once more.

4.5. Efficacy of $GABA_B$ Receptor Drugs on the Development of Preference in Wild Type and Mutant *Drosophila*

Administration of the $GABA_B$ receptor antagonist CGP 54626 for 24 hours prior to the first preference assay, affected the development and maintenance of preference to ethanol in the wild type *Drosophila* (Figure 3.8a). For the first and second day, whilst visually lower than the control preference index for the corresponding day, there is no statistical significance when compared to the treated flies. The statistical significance interestingly becomes apparent when the CAFE assay is conducted for the third time (day 3): the preference index is significantly different ($P<0.001$) for all three antagonist concentrations when compared to the control. This data therefore suggests that there is a delay in the antagonist taking affect in the wild type fly line.

The $GABA_B$ R1 KO appears to be more sensitive to the effect of the antagonist as the results differ from the control from the second day of the preference assay (Figure 3.8b). Whilst this is difficult to understand, this could indicate that the R2 receptor is very responsive and plays a big part in preference development. Another explanation could be that the antagonist CGP 54626 is working through an alternative receptor/mechanism and this would be strengthened by the results that show when the GABA receptor 1 subunit is knocked out, this potentiates the effect of the agonist (Figure 4.2).

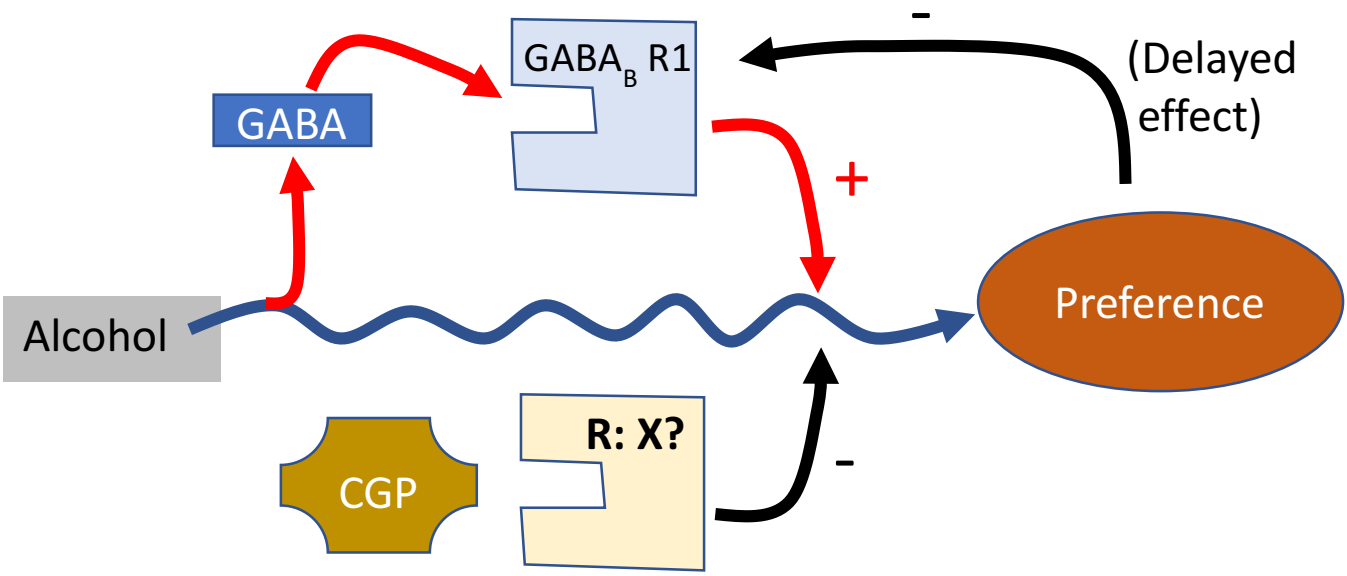


Figure 4.2: Theoretical dual mechanism of action for CGP 54626 antagonist in conjunction with the $GABA_B$ receptor 1 subunit.

CGP 54626 is a $GABA_B$ receptor antagonist that when administered could be mediated via an unknown receptor (labelled X). This would explain that when removing the GABA receptor 1 subunit, this potentiates the effect of the agonist.

On the other hand, whilst the $GABA_B$ R2 KO had no significance in the presence of the $GABA_B$ receptor agonist or antagonist with the two tolerance administration assays, there appears to be some behavioural preference changes in the presence of the antagonist. This indicates that this fly line is less sensitive to the ethanol exposures in the presence of the drug, indicating a potential importance of the receptor 1 subunit.

The agonist SKF 97541 administration has similar effects to the antagonist administration for all fly lines (Figure 3.9). For the wild type fly line, there is a delayed action and effect to the agonist with a notable change not occurring until the third day of the preference assay (Figure 3.9a). The most noticeable change as a result of the agonist administration appears in figure 3.9b with the $GABA_B$ R1 KO. This fly appears to have a negative preference to alcohol on day 1 but then shows a preference to ethanol on day 2. This occurs before day 3, when there is then a significant ($P<0.001$) decrease in preference for ethanol when compared to the untreated control for this day. This could indicate that the $GABA_B$ receptor agonist SKF 97541 works on both the $GABA_B$ receptor 1 subunit and

another receptor (Figure 4.3). Whilst this is a confusing phenomenon to explain, it is possible that the drug works through an unknown receptor/s and the $GABA_B$ as on day 2, the drug shows to not alter the preference index in the R1 KO line (Figure 3.9b) and on day 3, there is a sudden decrease in the preference index. This shows that on day 3, the drug is no longer bound to the unknown receptor but to just the receptor 1 subunit. Again, this discussion and results shown highlight the potential importance of the receptor 1 subunit in the development of preference.

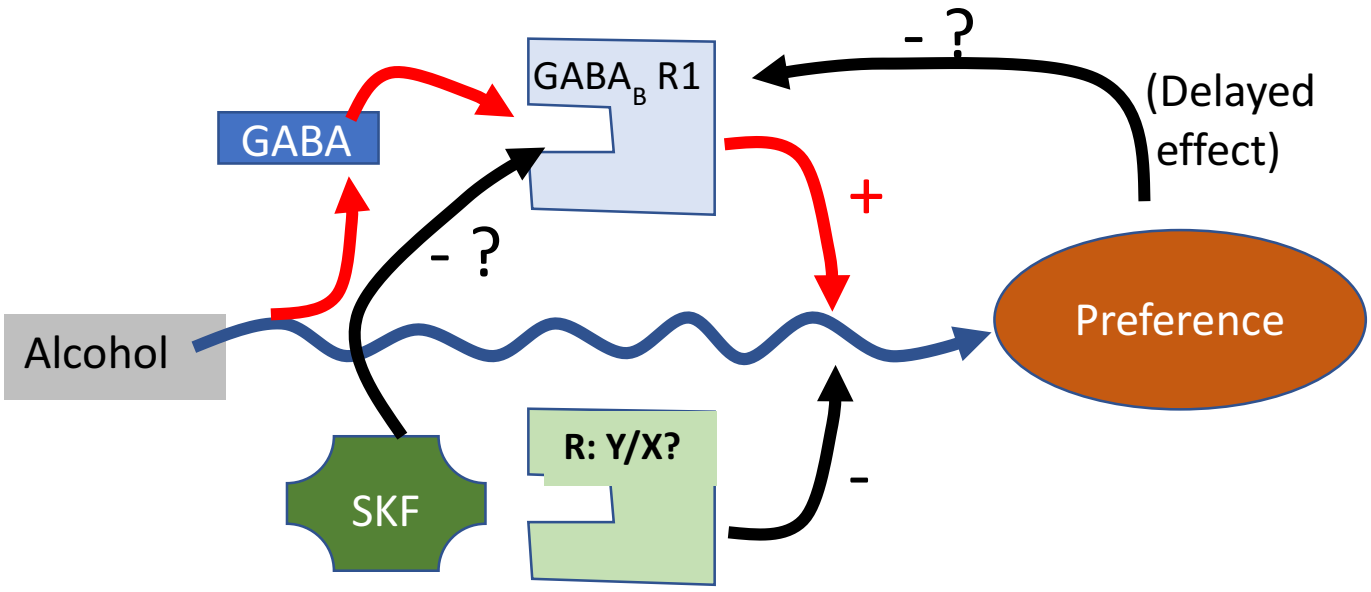


Figure 4.3: Theoretical dual mechanism of action for SKF 97541 agonist in conjunction with the $GABA_B$ receptor 1 subunit.

SKF 97541 is a $GABA_B$ receptor agonist that when administered could work through both an unknown receptor/s (Receptor X/Y) and the $GABA_B$ receptor 1 subunit. This is because, on day 2, the $GABA_B$ R1 KO does not respond with reducing preference and has a delayed effect.

4.6. Project Findings

When a KO mutant fly line causes a behavioural change, it indicates that the gene is directly or indirectly involved in the development of the behaviour. If there is no change however, it suggests that the gene is either not involved or its action can be compensated by another gene or mechanism of action.

By looking at the results shown in chapter 3, and discussions within sections 4.3, 4.4 and 4.5, it can be said that both the $GABA_B$ receptor antagonist CGP 54626

and $GABA_B$ receptor agonist SKF 97541 appear to have a similar mechanism of action on the wild type and mutant *Drosophila* used within this project whilst examining tolerance in the presence of ethanol. Pin and Prezeau (2007) have reported partial agonist activity when exploring the mechanism of action of the $GABA_B$ receptor for therapeutic interventions reporting similar partial agonist actions to those described within this thesis. It is suspected that the partial agonist would be an agonist with lower efficacy than the endogenous agonist and thus by competing with the endogenous agonist, the partial agonist will have a similar effect as an antagonist and this correlates with results shown and described.

The results portrayed and reviewed in sections 3.4 and 4.3 are hard to explain regarding the tolerance assay results described. The confusion occurs as a result of a drug administered at a lower concentration having more of a behavioural change than a drug administered at a higher dose. As shown in figure 4.1, one possible logical explanation is that at lower concentrations, the high affinity binding site initiates 'effector 1' that is responsible for increasing the ST50. This is compared with higher drug concentrations, whereby the drug binds to a low affinity binding site that induces 'effector 2' and this is responsible for decreasing the ST50 and subsequently, interfering with the equilibrium of the tolerance mechanisms. It can be thought from looking at this preliminary data however, that the $GABA_B$ R2 subunit plays a role in tolerance development in the *Drosophila.*

The preference assay results analysed in sections 3.7, 3.8 and 4.5 are equally as difficult to explain as the tolerance behaviours. The $GABA_B$ R1 KO appears to be more sensitive to effect of the administered CGP 54626 antagonist and the SKF 97541 agonist, and this potentially highlights the importance that the $GABA_B$ receptor 1 subunit has to play in preference development. It could also be suspected that the ligands are working through another receptor in addition to the R1 subunit. This development process is by no means simple and it would be naïve to think that one receptor has the sole responsibility of preference development. However, this data can be used as the foundations going forward for further research and thoughts.

4.7. Future Works

Throughout this project, the validity of the two mutant fly lines has been a concern. The mutant flies were dispatched to the university from Bloomington *Drosophila* Stock Centre (BDSC), a reputable depository located at Indiana University, USA, that has been used worldwide as a depository since 1913. More recently, the BDSC in 2016 was responsible for holding 59,126 *Drosophila* fly stock lines and distributed 217,072 subcultures (Bloomington Drosophila Stock Center 2017). It is thought therefore that the fly is what it is described as by the centre. After discussions with four laboratories (Both in the UK, and USA), it has been brought to the groups attention that whilst it is not common practice to take the time and make the effort to validate the work carried out by the BDSC, if interesting results have been obtained, it is sometimes worthwhile to carry out internal molecular assays to investigate this further. As described in chapter 2, a polymerase chain reaction was carried out and repeated a few times but was unsuccessful. It is not known at what stage the assay was unsuccessful; in the primer design or with the execution of the PCR itself but regardless, further refinement is needed as going forward with validating the results, it is of definite benefit to validate the KO subunits in each mutant and also to inspect that there is indeed a fully functioning receptor in the wild type. It is also extremely important to note that each mutant fly line has the same genetic background as one another and has therefore been genetically modified from the same wild type fly line.

Continuing with this project and contributing to literature available, it would be useful to continue the preference and tolerance assays over a greater number of days to investigate the duration of the drug effect. If the effect of the drug were to continue and be significant over more consecutive days, it would indicate that from results in chapter 3, that there have been some lasting changes in the *Drosophila* that would need to be investigated and described further. It would also be of interest to determine the longevity of the drug and to also determine if there are any permanent effects of the drug affecting this receptor or otherwise.

In addition, it would be wise to repeat the experiments a greater number of times to ensure a quality cohort of results and data obtained. This will ensure clear and confident statements can be made and presented. It is a great shame that time restraints limited a wider range of concentrations from being used for this project

as it would have been a great benefit. It would also be interesting to create a greater number of variables with regards to ethanol exposures. Maximising the number of days for the preference and tolerance assays would have given useful data and information into the behaviours of the addiction behaviours and the functions of the drugs but it is felt that this project has reinforced the evidence that the $GABA_B$ receptor contributes to addiction related behaviours and it has provided further compulsion towards unravelling $GABA_B$ receptor complex role. It is hoped that with a greater understanding of the $GABA_B$ receptor function amongst other systems, it will be possible to have an impact of further alcohol related morbidity and mortality.

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A Gut Feeling about GABA: Focus on $GABA_B$ Receptors

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Appendices

Appendix 1.1: A table to highlight data used regarding number of alcohol related deaths from 2001 to 2015 categorised by age. Taken and modified from the Office for National Statistics (2017) publication titled '*Alcohol-related deaths in the UK: registered in 2015'.*

Year/Sex	Age	<1	01-04	05-09	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85+
1994	Male	2	0	1	0	2	16	40	92	163	253	352	353	351	344	343	276	123	99	39
	Female	0	2	0	1	1	3	13	35	82	147	202	167	208	197	197	174	117	79	51
1995	Male	3	0	0	0	5	10	35	105	169	290	421	377	420	410	341	290	172	86	48
	Female	0	0	1	0	0	2	11	48	96	148	211	226	211	211	203	207	141	83	48
1996	Male	1	1	0	1	1	14	26	113	223	323	480	494	445	434	337	275	175	78	36
	Female	0	2	0	0	1	4	11	41	98	189	235	252	216	230	213	176	143	87	51
1997	Male	0	1	1	2	5	14	29	112	238	386	542	594	484	405	407	319	186	84	47
	Female	1	2	1	0	2	6	17	41	118	173	264	279	235	242	219	215	153	84	68
1998	Male	0	1	0	0	8	19	40	132	255	459	545	663	526	462	396	312	198	103	53
	Female	1	2	1	0	1	2	15	43	128	181	229	316	295	266	251	176	152	91	47
1999	Male	1	0	0	0	5	15	43	143	261	439	551	699	606	514	423	296	203	99	63
	Female	1	0	0	0	2	6	19	58	153	225	319	303	303	240	224	194	168	81	64
2000	Male	0	0	0	1	5	12	39	129	253	448	579	726	644	569	410	311	209	96	52
	Female	0	1	0	0	2	4	34	63	134	221	285	350	316	262	245	196	158	79	51
2001	Male	0	0	0	0	10	18	46	148	283	493	645	823	687	568	480	316	233	122	66
	Female	0	0	0	1	1	3	16	66	139	233	331	395	319	293	238	193	170	90	73
2002	Male	0	0	0	0	3	16	50	146	282	505	676	772	745	618	490	348	228	118	72
	Female	0	0	0	0	0	3	21	57	125	268	347	363	335	302	258	220	138	113	82
2003	Male	1	0	0	1	3	12	45	128	326	531	710	841	836	666	542	373	228	142	58
	Female	0	0	0	0	3	4	27	64	154	244	321	363	399	325	265	199	187	98	68
2004	Male	1	0	0	0	3	14	51	130	296	530	704	834	835	699	550	352	249	135	48
	Female	0	0	0	1	2	4	23	60	174	245	340	407	390	316	258	222	160	116	72
2005	Male	0	0	1	0	9	16	36	131	292	497	759	855	877	735	518	391	231	152	66
	Female	0	0	0	0	1	6	21	65	159	243	380	391	428	308	262	191	161	127	77
2006	Male	0	0	0	0	2	11	50	128	345	541	775	878	886	788	560	383	209	146	66
	Female	0	0	0	1	2	5	31	53	156	290	389	403	441	375	274	227	157	110	76
2007	Male	1	0	0	0	1	10	53	139	305	572	703	909	911	805	554	346	214	135	74
	Female	0	0	0	1	0	7	30	74	154	287	354	429	411	423	265	210	166	107	74
2008	Male	0	0	0	1	5	22	49	154	336	577	759	918	897	845	574	423	237	134	68
	Female	0	0	0	0	0	6	17	75	147	263	362	452	437	408	296	221	160	109	79
2009	Male	0	1	0	0	2	18	56	133	296	627	737	809	812	823	551	340	264	134	87
	Female	0	0	0	0	3	4	33	78	156	266	360	413	407	392	267	224	174	100	97
2010	Male	0	0	0	1	5	16	59	139	284	544	780	830	882	873	621	388	234	124	85
	Female	0	0	0	0	3	3	25	85	164	265	350	411	418	380	282	206	132	119	82
2011	Male	0	0	0	0	2	8	54	129	265	548	739	805	838	871	612	431	254	144	92
	Female	0	0	0	0	0	7	32	82	161	291	334	426	411	406	250	212	168	95	81
2012	Male	1	0	0	0	0	6	49	124	252	488	695	729	765	757	655	372	308	178	94
	Female	0	0	0	0	0	6	22	71	98	241	367	386	413	357	331	217	174	112	99
2013	Male	1	0	0	0	2	7	45	118	252	501	693	815	810	786	677	349	263	152	94
	Female	0	0	1	0	2	4	19	67	124	227	343	405	407	405	309	174	154	119	91
2014	Male	0	0	0	0	3	5	39	94	246	414	711	794	809	819	743	442	283	182	103
	Female	0	0	1	0	1	6	25	86	126	230	375	416	430	366	319	224	206	112	87
2015	Male	0	0	0	0	0	4	35	110	232	413	690	831	823	770	769	440	294	164	118
	Female	0	0	0	0	0	3	21	63	134	252	379	400	462	396	336	245	171	118	85

Appendix 1.2: A table to highlight data used regarding number of alcohol related deaths from 2001 to 2015 categorised by condition. Taken and modified from the Office for National Statistics (2017) publication titled '*Alcohol-related deaths in the UK: registered in 2015*'.

ICD Description of Condition/Sex/Year	Sex	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Mental & behavioural disorders due to alcohol use	Male	338	306	2002	2002	401	349	322	437	424	446	300	321	321	339	288
	Female	147	123	114	135	138	158	164	203	172	182	127	121	129	150	117
Degeneration of nervous system due to alcohol	Male	7	6	12	7	4	2003	2003	2	6	3	8	4	5	12	6
	Female	1	2	2	3	2	0	4	4	3	1	5	3	3	1	3
Alcoholic polyneuropathy	Male	0	0	0	0	0	1	1	0	2004	2004	3	0	0	0	0
	Female	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Alcoholic cardiomyopathy	Male	94	94	88	78	59	74	66	68	78	63	93	2005	2005	68	62
	Female	13	29	11	16	16	9	10	12	20	10	13	14	16	16	14
Alcoholic gastritis	Male	1	4	0	1	0	3	6	2	3	4	6	1	3	5	2006
	Female	2	0	2	1	1	1	3	1	1	0	3	1	0	0	2
Alcoholic liver disease	Male	2148	2278	2514	2465	2609	2768	2816	2968	2755	2881	2946	2651	2786	2845	2878
	Female	1091	1123	1192	1303	1275	1391	1441	1434	1403	1397	1499	1429	1404	1488	1533
Chronic hepatitis, not elsewhere classified	Male	21	16	14	14	12	13	10	16	23	6	0	0	4	1	2
	Female	48	57	44	49	46	55	58	45	47	44	3	4	6	4	1
Fibrosis and cirrhosis of liver	Male	859	835	905	900	868	918	860	827	874	871	874	910	871	911	971
	Female	548	571	598	558	555	571	564	538	555	525	510	569	567	609	602
Alcohol induced chronic pancreatitis	Male	19	24	22	34	43	33	35	39	29	37	5	8	6	9	7
	Female	14	9	10	9	9	8	13	9	12	17	0	1	5	2	0
Accidental poisoning by and exposure to alcohol	Male	90	70	86	91	100	97	106	110	117	120	257	249	235	242	205
	Female	36	42	41	39	51	53	51	44	51	53	103	118	125	127	108
Intentional self-poisoning by and exposure to alcohol	Male	0	1	2	2	2	3	1	1	0	3	1	3	6	1	3
	Female	1	1	0	0	0	0	1	2	2	0	4	1	2	1	1
Poisoning by and exposure to alcohol, undetermined intent	Male	1	1	4	4	5	4	5	6	6	5	10	4	4	0	3
	Female	0	0	1	1	1	0	1	3	1	1	5	2	3	0	1