Mechanisms Of Naltrexone-induced Reduction of Ethanol Preference In *Drosophila melanogaster*

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I. ABSTRACT

Naltrexone is an opioid antagonist used to treat alcohol dependence in human beings ever since its approval by Food and Drug Administration in 1994. Naltrexone exerts its action by blocking on central opioid receptors that mediate the drinking or reward behaviours, thus reducing the alcohol consumption. Although various animal and clinical studies have demonstrated the efficacy of Naltrexone, its action on reducing the preferential ethanol consumption in *Drosophila melanogaster* has not been illustrated so far. So it was of our interest to demonstrate the effect of Naltrexone on the drinking behaviour in fruit flies and to further explore the molecular mechanisms underlying this effect. In our study, we have employed the well-established CAFE methodology to test the preference of flies to consume alcohol food over normal food. 1-3 day old male flies (wild type) were used for all the experiments which were exposed or unexposed to 15% ethanol to examine the preferential consumption. Preference assays were conducted with or without Naltrexone treatment to demonstrate its effect under various experimental conditions. In addition to the behavioural assay, we have attempted a biochemical estimation to observe the changes in the phosphorylation patterns of protein kinase C (PKC) using an ELISA-based PKC kinase activity assay in order to explore the mechanism of action of Naltrexone in relation to PKC which has been identified to mediate alcohol addiction processes. To further explore any PKC-mediated mechanism of Naltrexone effect, preference assays were conducted in *Drosophila* PKC mutant line-20790. Our results showed that *Drosophila* pre-exposed to ethanol, prefers to consume ethanol food over non-ethanol food and for the first time we have demonstrated that Naltrexone reverses this preference to consume ethanol food. Our data also shows that mechanism of Naltrexone effect appears to be independent of PKC-mediated pathway and we propose that Naltrexone might be operating through a different system (eg; pathways or receptors or signalling molecules associated with neural circuitry such as Dopamine) and more research is needed to explore these mechanisms in detail to develop new hypotheses on potential therapeutic targets.

II. ACKNOWLEDGEMENTS

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Last but not the least, I am always greatly thankful to my family, who believe in me and support my decisions to achieve higher goals in my life.

III. LIST OF FIGURES

IV. ABBREVIATIONS

CAFE- Capillary Feeder

PKC- Protein Kinase C

µl- microliter

mM- Millimolar

mg- milligram

2h- 2 hours

24h- 24 hours

48h- 48 hours

Unexposed- Drosophila flies that are not pre-exposed to ethanol

Exposed- Drosophila flies that are pre-exposed to ethanol

INTRODUCTION

Drug addiction, also called substance use disorder, is the excessive use of a drug to the point of compulsive drug consumption and drug seeking. The American Psychiatric Association's latest edition, DSM-V (The Diagnostic and Statistical Manual of Mental Disorders), has combined categories of DSM-IV, substance abuse and substance dependence into single term as 'substance related disorder'. It recognizes substance related disorders caused by ten different classed of drugs such as caffeine, alcohol, hallucinogens, opioids, tobacco, cannabis, and cocaine etc. Substance related disorders are classified into as Substance use disorder and Substance-induced disorder. Substance use disorder can be defined as patterns of symptoms developed as a result of continuous and excessive consumption of the substance and Substance-induced disorder include withdrawal, intoxication, substance induced anxiety and depressive disorders (http://addictions.about.com/od/aboutaddiction/a/Dsm-5-Criteria-For-Substance-Use-Disorders.htm, 2016).

Alcoholism also called alcohol use disorder **(AUD)** or alcohol dependence is a substance use disorder in which an individual is physically or psychologically addicted to alcohol despite of the social and health problems associated with persistent and excessive alcohol consumption. Alcohol addiction remains one of the most widely spread addiction and alcohol abuse is a global health problem with major social, mental, and economic consequences (Liang and Olsen, 2014) In 2012, about 3.3 million deaths accounting for 5.9% of all deaths were reported by World Health Organization. About 9% of men in the UK and 4% of UK women show signs of

alcohol dependence according to NHS statistics. Alcohol abuse has been the second most common mental disorder worldwide (Robins and Regier, 1991). Till date, there is no therapeutic approach which can satisfactorily solve these problems, therefore understanding the mechanisms underlying alcohol addiction has been an important biomedical goal for the scientific community which aims to develop safe and effective pharmacological therapies.

1.1 Factors affecting alcoholism

Alcohol is a psychoactive substance which is capable of producing dependence and addiction behaviours. Alcohol addiction is a complex disease with genetic and environmental factors having a major role in its development (Flatscher-Bader and Wilce, 2009). Various factors have been identified to affect alcoholism including age; gender; environmental- factors such as culture, economic development and ethnicity (Chartier and Caetano, 2010). Genetic-heritable familial risk factors account for a major portion of variability in alcoholism although individual genes responsible for alcoholism are yet to be identified (Merikangas et al., 1998). Socio-economic studies have suggested that there are a proportionally larger number of drinkers, and drinking events among the higher socioeconomic status and these tend to be more in the low-risk drinking patterns than among the lower socioeconomic group who are more vulnerable to the harmful consequences of alcohol (Grittner et al., 2012).

1.2 Mechanisms underlying alcoholism

The molecular mechanisms underlying alcohol consumption/addiction are still not completely understood, but a significant insight into them has been provided by mammalian studies conducted in mice employing genetic modification methods such as gene knock-in and knock-outs, gene overexpression and down regulation. The

majority of these studies have focused on genes expressed in brain which widely range in their function including neurohormones, neurotransmitters and various signaling molecules and have been implicated in relation to ethanol (Crabbe et al., 2006, Worst and Vrana, 2005)

Most of the addictive drugs, including alcohol, target the dopaminergic corticolimbic system of the brain that is involved in the regulation of cognition, emotion, motivation, movement, and feelings of pleasure. This system which rewards our natural behaviours upon overstimulation by the addictive drugs results in euphoric effects that teaches people to repeat the behaviour and alcohol has shown to have many potential targets in this brain region including ion channels and membranes (Flatscher-Bader and Wilce, 2009)..

Interaction of alcohol with multiple neurotransmitters and neuromodulators affects functionality of the brain and causes numerous physiological effects, for instance, alcohol exposure can disrupt the equilibrium between inhibitory γ-aminobutyric acid (GABA) and excitatory (Glutamate) neurotransmitters in the central nervous system which ultimately leads to development of tolerance and dependence (Liang and Olsen, 2014).

Within the mesolimbic dopaminergic system, acute ethanol exposure inhibits the glutamatergic and increases the GABAergic neurotransmission while chronic ethanol exposure has an opposite effect (Vengeliene et al., 2008). The significant effect of alcohol on the GABA neurotransmission can be observed in the figure below (Roberto et al., 2004, Gilpin and Koob, 2008)

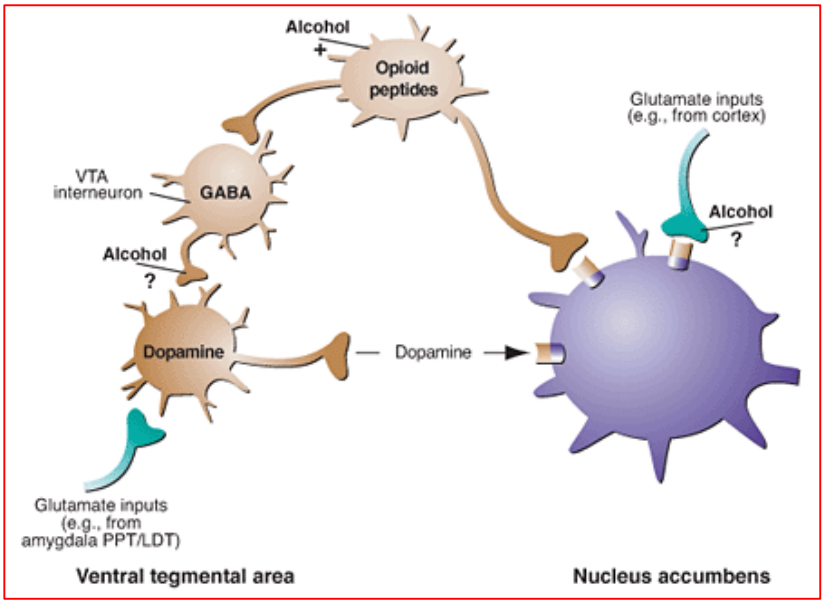

Figure 1. Effect of alcohol on the neurotransmitter systems of the brain's reward pathways. In the VTA, ventral tegmental area, alcohol may inhibit the GABAergic transmission, which in turn activates dopamine in VTA resulting in dopamine release by neurons that leads to the activation of reward processes. In a similar manner alcohol may inhibit glutamate (excitatory neurotransmitter) release from nerve terminals.

Ethanol interacts with various receptors widely distributed in the central and peripheral nervous systems, changes their physiologic effects and ultimately results in addiction behaviours hence it is critical to understand the neurobiological mechanisms underlying the alcohol addiction to develop new pharmaceutical therapies and effective strategies for treatment.

1.3 Role of endogenous opioid system in alcoholism

As discussed earlier, alcohol interacts with various neurotransmitters systems to produce a range of pharmacological effects. Among these, the endogenous opioid system has been shown to play an important role in mediating alcohol dependence/addiction (Herz, 1997, Gianoulakis, 2001). There are three types of

opioid receptors namely, mu, kappa and delta opioid receptors and their respective opioid peptides are β-endorphin, dynorphin and enkephalin. It has been demonstrated that alcohol directly or indirectly interferes with the reward pathways mediated by these receptors leading to the development of addictive processes(Herz, 1997).

Few studies suggested that alcohol may induce the release of certain opioid peptides which could interact with reward centers of the brain leading to further consumption of alcohol (Koob et al., 1998, Spanagel et al., 1992, Acquas et al., 1993).

Acute ethanol administration has been shown to increase β-endorphin levels and this elevation was found to be greater in alcohol-preferring animals (selectively bred rodents) than in alcohol-avoiding population (de Waele and Gianoulakis, 1993). Consistent with this data, further studies conducted in humans also reported an increase in β-endorphin levels in the nucleus accumbens region of the brain after alcohol consumption (Mitchell et al., 2012, Nutt, 2014).

In addition to endorphin, the other endogenous opioids, dynorphin and enkephalin were also known to be affected by alcohol. In rodents, acute alcohol exposure has shown to increase dynorphin levels in the nucleus accumbens and central nucleus of amygdala regions of the brain (Marinelli et al., 2006, Nutt, 2014).

Blocking the opioid system by administering opioid antagonists has been shown to reduce ethanol consumption in alcohol dependents. Naloxone, was the first experimental opioid antagonist used to treat alcoholism and can reduce alcohol consumption in rodents (Reid et al., 1991) and similar antagonists were developed namely Naltrexone and Nalmefene.

1.4 Effects of ethanol on PKC

Protein kinase C (PKC) isozymes are a group of serine-threonine kinases which have been recognized as an important family of enzymes that regulate various physiological

aspects of nervous system such as neurotransmitter synthesis and release; neuronal development, excitability, gene expression and signal transduction; receptor and ion channel function. Many studies have evidenced the role of PKC in the effects of various abused drugs and their corresponding behavioural responses (Olive and Messing, 2004).

Pharmacological studies on drugs that inhibit or activate PKC isozymes have identified critical role of PKC in regulating GABA(Gamma –amino butyric acid) receptor function in response to ethanol, neurosteroids and benzodiazepines (Song and Messing, 2005).

PKC has been implicated in addiction behaviours and it has been shown that ethanol mediates the phosphorylation of conventional PKC, which is needed for its catalytic activity. Studies conducted in mice have demonstrated a rapid increase in phosphorylation of PKC after acute ethanol exposure in specific regions of mammalian brain (Wilkie et al., 2007). Data from other studies have implicated the acute and chronic effects of ethanol on the activity of PKC isozymes and their further downstream effects, for instance, ethanol utilizes PKCδ to alter the activity of (AC7) adenylyl cyclase type7 (Nelson et al., 2003) and a PKCδ-ethanol dependent mechanism was observed to mediate the upregulation of the L-type Ca^{2+} ion channel function (Gerstin et al., 1998).

Differential effects of ethanol exposure on the activity of PKC and its specific isoforms in brain have been explored by studies conducted in rats. Acute ethanol administration was shown to alter the synthesis and translocation of PKC in brain in an isoform and brain region specific manner which in turn alters the serine phosphorylation of GABA and NMDA receptors, whereas chronic administration of

ethanol prevented ethanol-induced alterations in PKC expression in the P2 fraction (Kumar et al., 2006).

Research findings suggest that PKC could be a potential target for drugs to curb excessive alcohol consumption since deletion of PKCγ gene produced high drinking mice phenotype which require excessive levels of alcohol to reach intoxication. This models the human phenotype with a lowered risk of developing alcohol addiction (Newton and Ron, 2007).

1.5 Drosophila as a model to study alcohol addiction

Drosophila melanogaster, commonly known as fruit fly, has been one of the extensively used animal models in biological research to gain insight into cellular, molecular, developmental and disease processes that are conserved in mammals, including humans. The homology of about 75% of human disease genes with *Drosophila* genome suggests that flies can be an effective model for studying wide range of human disease genes including but not limited to Parkinson's and Alzheimer's diseases (Adams et al., 2000, Reiter et al., 2001).

Drosophila, within its natural environment, encounters high levels of ethanol and show remarkable similarity with mammals in the way they metabolize ethanol and also in their behaviours after ethanol exposure. These parallels support the use of this animal for studying alcohol addiction and may provide information on potential drug target to treat alcoholism in human beings.

Studies on alcohol addiction behaviours in *Drosophila* have demonstrated interesting similarities between human beings and fruit flies in the way they prefer and self-administer to ethanol, overcome the aversive stimulus in order to consume ethanol

and return to ethanol consumption at a high level following a period of abstinence. (Devineni and Heberlein, 2009).

1.6 Naltrexone for the treatment of Alcohol dependence

Naltrexone is an opiate antagonist that has been reported to reduce alcohol craving. Its main effect is on blocking central opioid receptors. Food and Drug Administration, United States, approved naltrexone in 1994 for the treatment of opioid addiction. By blocking opioid receptors that mediate the drinking behaviour, it has been shown that naltrexone reduced alcohol consumption. Clinical trials data suggested that a dose of 50mg per day was sufficient to reduce the relapse rates in alcohol dependent patients (Morris et al., 2001).

Naltrexone hydrochloride is a specific long lasting opioid antagonist which attenuates or completely reverses the effects of opioids administered intravenously. Naltrexone acts on these receptors which are located in central and peripheral nervous system. Initially naltrexone was approved by FDA for the treatment of addiction to opioids such as morphine, cocaine and oxycodone. Clinically it is now more frequently used to treat patients with alcohol misuse but its mechanism of action in treating alcoholism has not been completely elucidated (Adams et al., 2000).

Naltrexone exerts its antagonistic effect by blocking the opioids at the μ-opioid receptors resulting in reduced dopamine release and thus may attenuate the rewarding effects of ethanol (Heinz et al., 2005). This blockade is competitive and the degree of blockade depends on the affinity for the receptors and the relative concentration of agonists to antagonists. The mechanism of action of naltrexone can be diagrammatically represented as below

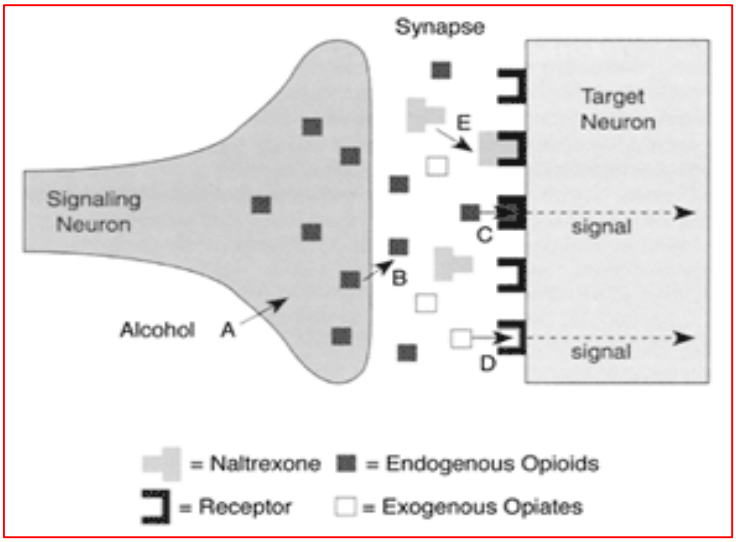

Figure 2: Mechanism of action of Naltrexone (Volpicelli et al., 1992)

A-Stimulation of release of endogenous opioids by alcohol that might produce the euphoric feelings

B-Release of Endogenous opioids into the synapse

C-Stimulation of opioid receptor activity that produces target neuronal signal

D-Morphine (exogenous opioid) may also stimulate opioid receptors

E- Blockade of opioids at opiate receptors by Naltrexone

Naltrexone reduces the drinking desire and the amount of alcohol by alcoholic subjects by supressing the craving for alcohol. It is hypothesized that naltrexone may reduce drinking via suppressing craving for alcohol and that this effect may be related in part to naltrexone's ability to activate the hypothalamo-pituitary-adrenocortical axis (O'Malley et al., 2002).

Naltrexone has advantages such as, it is easy to administer (orally), safe, well-tolerated, and does not have addictive potential; and tolerance does not develop to the opioid antagonism. It has a half-life of about 4-9h and its active metabolite 6-β-naltrexol has a longer half-life of 12-18h (Davidson et al., 1996). However it has some side effects which limits its effectiveness such as fatigue, dysphoria, nausea and development of withdrawal symptoms (Oncken et al., 2001)

Preclinical studies in rhesus monkey (Williams and Woods, 1999) and mice have demonstrated that naltrexone reduced the alcohol drinking of the animals (Sinclair, 2001). However, no studies have been published till date which illustrated the efficacy of Naltrexone in reducing the alcohol preference in fruit fly, *Drosophila melanogaster,* which displays high similarity to mammals with regards to alcohol related behaviours. Our study aimed to test the effect of naltrexone on *Drosophila*'s preference to alcohol and explore the underlying mechanisms which may provide information to build new hypotheses to study alternative mechanisms involved in alcohol addiction that could be a potential therapeutic target!

Main Aim

To determine the mechanisms by which naltrexone inhibits alcohol induced behaviours in Drosophila

Objectives

- To validate the CAFE assay as a method for measuring the effect of pharmacological interventions in alcohol induced behaviours
- To determine whether naltrexone reverse alcohol preferences in alcohol exposed flies
- To determine whether naltrexone affect PKC activity in alcohol exposed flies
- To determine whether PKC is required for the naltrexone effect.

1. Materials and Methods

Flies and Media: Flies used for all experiments were 1-3 day old male flies reared at 25ºC and 70% humidity on commercially available readymade mixed dried food (Batch no: B8A03876 obtained from Phillip Harris) which is a complete culture medium for *Drosophila*. Clean and autoclaved glass bottles were used for growing flies on a daily basis. Flies were sub-cultured every 7-10 day period.

Drosophila strains used for the experiments were wild type and mutant flies (**20790**-Bloomington line 20790 carrying a transposon insertion which alters the expression and function of the PKC isomer 53E; Stock ID: FBst0020790) obtained from Bloomington stock centre.

Liquid food used for the preference assays contained 5% sucrose and 5% yeast extract dissolved in distilled water with or without 15% ethanol (v/v), which was prepared fresh on the day of experiment.

Capillaries: Calibrated glass micropipettes with a total fill capacity of 5ul (catalogue no: CAP-TF-5 by Jaytec Glass Ltd), were used to introduce liquid food to the flies.

Naltrexone: Purified Naltrexone hydrochloride ((5α)-17-(Cyclopropylmethyl)-4,5-epoxy-3,14-dihydromorphinan-6-one hydrochloride), was obtained from Tocris Bioscience(catalog no: 0677).

PKC Kinase Assay Activity Kit: PKC Kinase Assay kit used for the analysis of PKC activity was obtained from Abcam (catalog no: ab139437).

2.1 CAFE Assay

Capillary feeder (CAFE) assay introduced by William and his colleagues, is a method which allows a precise and real time measurement of food consumption by *Drosophila melanogaster*. The visibly clear descent of meniscus on the capillaries allows simple, rapid and continuous measurement of food consumption. Using CAFE the amount of ingestion of food by individual or grouped flies can be measured ranging from minutes to entire lifespan (Ja et al., 2007)

CAFE assay is the major experimental method used for our study with different treatment regimens to demonstrate the preferential consumption of ethanol food by flies.

2.1.1 Preparation of the CAFE:

CAFE model comprises of a 1.5cm diameter plastic vial which is divided into two chambers: upper chamber and a lower chamber separated by a cotton plug (shown in Fig 3). The upper chamber contains flies and the lower chamber has a bottom pierced to allow entry of air and water vapour. Glass capillaries used to introduce liquid food to the flies in the upper chamber were inserted through the cap via truncated 200µl pipette tips. Flies were anesthetized using CO2 and introduced in to the upper chamber. Unless otherwise stated, for all preference assay experiments, 6 flies and four glass capillaries were inserted into each plastic vial and triplicates of vials were used for each experimental condition. To minimise the evaporation of food from the capillaries, a layer of vegetable oil was added to the top of capillaries. An empty plastic vial containing the glass capillaries with food but no flies was used as a control

for evaporation. Except during measurement of readings, all vials are maintained at standard 25°C and 70% humidity. Capillaries were replaced when needed.

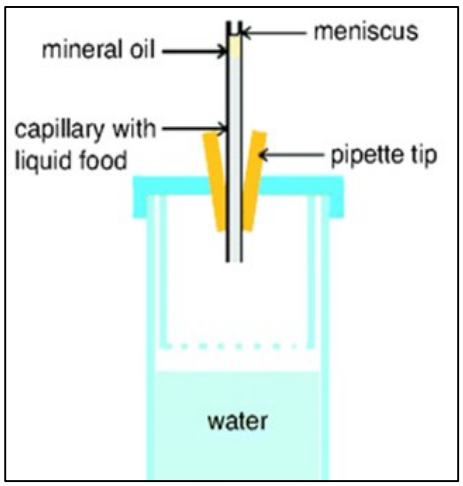

Figure 3: Schematic diagram of CAFE-Liquid food is introduced via the glass capillary through a truncated pipette tip. The inner chamber contains water and provides moisture. A layer of mineral oil is added on the top of the capillary to prevent evaporation.

2.1.2 Different Treatment regimens for Preference Assay:

I) Preference Assay

To demonstrate the preferential consumption of ethanol, flies were pre-exposed or unexposed to 15% alcohol for 48hours. Following a 24 hour starvation period, four capillaries - two capillaries with liquid food and another two capillaries with ethanol food were introduced into all the vials offering the flies the choice between the two types of food, as shown in Figure 2. Preference readings are noted after 2h and 24h. Food consumption by flies is quantified by measuring the descent/lowering of meniscus on the capillaries. Preference is calculated using the formula below:

$$PI = \frac{(Ethanol\ food\ consumption) - (Non\ ethanol\ food\ consumption)}{Total\ consumption}$$

Where

PI= Preference Index

Ethanol food consumption= (average of descent of meniscus of ethanol food)-(average of descent of meniscus in control of evaporation),

Non-ethanol food consumption= (average of descent of meniscus of non-ethanol food)-(average of descent of meniscus in control of evaporation) and

Total consumption= (Ethanol food consumption) + (Non-ethanol food consumption)

A preference index value ranges from -1 to +1, positive values indicate preference to ethanol and negative values represent no preference or repulsion to ethanol.

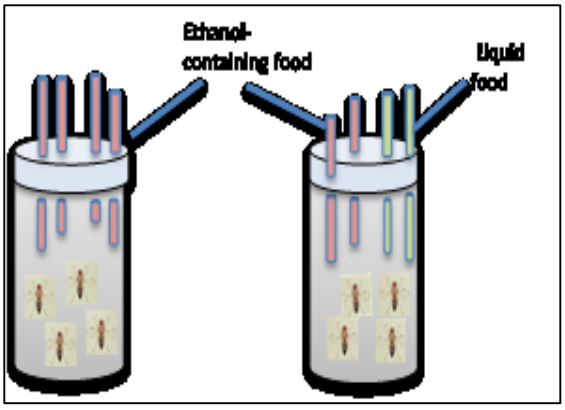

Figure 4: Preference Assay setup- Flies are fed with two types of food, ethanol food (represented by red capillaries) and liquid food (represented by green capillaries).

II) Naltrexone Treatment:

Flies were treated with Naltrexone hydrochloride, to observe any changes in the consumption/preference of ethanol. All flies were pre exposed to 15% ethanol for 48 hours fed through capillaries with varying concentrations of Naltrexone (0%, 0.05%, 0.1% and 0.5%) dissolved in liquid food for 24 hours. Following this treatment, flies were deprived of food for 24hours (starvation) and preference readings are noted after 2h and 24h and preference values were calculated as described above.

As control, unexposed flies (flies which were not pre-exposed to ethanol) were treated with the same concentration of Naltrexone to observe any changes in the alcohol consumption patterns by flies.

To further analyse the effect of Naltrexone the following procedures were also carried out:

a) Flies were exposed to 15% ethanol food for 48 hours and treated with 0.1% Naltrexone for 24h. The following day flies were fed with liquid food without ethanol for 24h and following a 24h starvation period, preference assay was performed.
b) Flies were exposed to 15% ethanol food for 48 hours and fed with liquid food without ethanol for 24 hours. This is followed by Naltrexone treatment for 24h and after 24h starvation period, preference assay was performed.
c) Flies were exposed to 15% ethanol food for 48 hours and fed with liquid food without ethanol for another 48h. Following a 24h starvation period, preference assay was performed. This group represents untreated condition (no naltrexone treatment).

Preference assay was carried out in Drosophila PKC-mutant line-20790 to further explore the reversal effect of Naltrexone in relation to PKC.

1.2 PKC Kinase Activity Assay

2.2.1 Background:

PKC Kinase Activity Assay was performed using the commercially available PKC Kinase Activity Kit to analyse the activity of PKC in solution phase. The kit is based on the principle of solid phase ELISA (enzyme-linked immune-sorbent assay). It utilizes a synthetic peptide which specifically acts as a substrate for PKC and a polyclonal antibody to recognize the phosphorylated form of the substrate. This assay

is a non-radioactive assay that allows rapid, reliable and safe method for both the quantification of the activity of PKC and screening of the activators inhibitors of PKC in partially purified or purified enzyme preparations from any species.

2.2.2 Preparation of samples and reagents for the Assay:

Samples:

Sample preparation for PKC Activity assay includes 3 groups of flies as follows

Unexposed flies- flies which were fed with liquid food without ethanol for 48h and untreated with Naltrexone

Exposed flies- flies pre-exposed to 15% ethanol food for 48h followed by 24h food to act as a control for Naltrexone treatment.

Treated flies- flies pre-exposed to 15% ethanol food for 48h and treated with 0.1% Naltrexone for 24h

Lysis Buffer: Lysis buffer was prepared by mixing 20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride (PMSF).

On the day of assay, following the above described exposure, flies were transferred from the plastic vials to 1.5ml microfuge tubes labelled according to their respective treatment groups. All flies were sacrificed in liquid nitrogen and fly heads were separated using a vortex machine. The heads were then homogenised in 80µl of Lysis buffer using sterile plastic pestles for Eppendorf tubes. The samples were then

centrifuged at 10,000xg for 5 minutes using a microcentrifuge and the supernatants were transferred to fresh pre-chilled microfuge tubes and maintained on ice.

2.2.3 Assay procedure

The assay procedure was performed according to the manufacturer's instructions (Abcam) as follows:

All the samples, standards and reagents were prepared

↓

Wells soaked with Kinase Assay dilution buffer (provided with the kit) at room temperature for 10 minutes

↓

Buffer was aspirated and samples were added to appropriate wells

↓

ATP (provided with the kit) was added to all the wells to initiate the reaction

↓

Incubation at 30°C for 90 minutes

↓

Kinase reaction was terminated by emptying the wells

↓

Phosphospecific Substrate Antibody (provided with the kit) was added to each well

↓

Incubation at room temperature for 60 minutes

↓

Wells were washed thrice with wash buffer (provided with the kit)

↓

TMB substrate (provided with the kit) was added to the all wells

↓

Incubation at room temperature for 30-60 minutes based on the colour development in the wells.

↓

Stop solution was added and absorbance measured immediately

2.2.4 Protein Estimation:

Protein concentration was measured using a Bradford reagent (Bio-Rad). A bovine serum albumin (BSA, Sigma) standard curve was carried out by preparing a solution of 1mg/ml which was serially diluted. All samples (50µl) were pipetted into a microtiter plate, Bradford reagent was added to all of the samples including the unknown protein samples, Bradford reagent (150µl) was added and after 10 min absorbance was read at 595nm using a multimode plate reader. PKC specific activity was expressed as PKC absorbance divided by protein concentration.

2. RESULTS

3.1 Preference Assay

In order to validate the CAFE preference assay a set of experiments were carried out to demonstrate the effect of ethanol pre-exposure on the preference behaviour of the flies. Flies were pre exposed for 48 hours to either food or ethanol-containing food and following a 24 starvation period they were subjected to the CAFE assay. Figure5 shows that flies that were pre-exposed to 15% ethanol showed preference to consume ethanol food over normal food. In figure 5, the dot-bars indicate preference index (PI) values of unexposed flies and checker-bars represent ethanol exposed flies (48h). It can be observed that pre-exposed flies showed higher preference than unexposed flies after 2h time point and this preference was increased over a 24h time period.

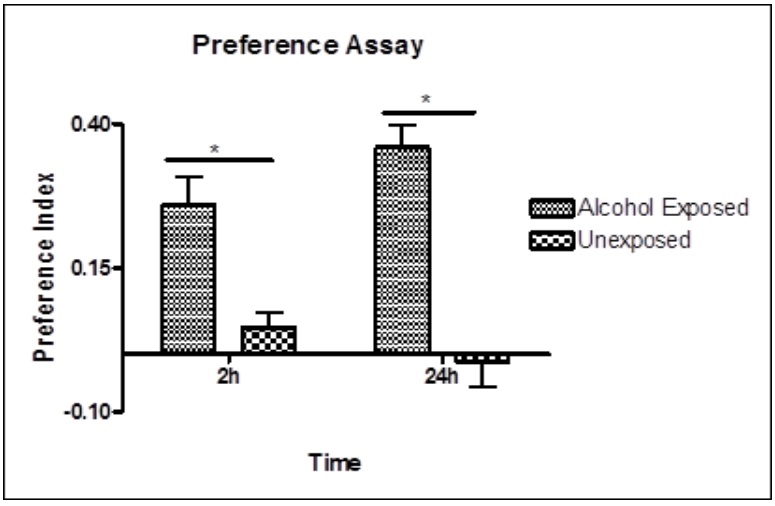

Figure 5: Preference assay carried out for 2h and 24h time periods following 48h exposure to either food or food plus ethanol. Data is the average of 2 experiments, each with three vials for both exposed flies and unexposed flies and containing 6 flies each (n=6, the number of vials). The data is significant according to the statistical analysis using student t-tests where $P<0.05$ with Mean ± SEM of unexposed group: 0.01633 ± 0.03033 and exposed: 0.3099 ± 0.04878.

3.2 Naltrexone treatment

In order to test whether the drug Naltrexone which is used for the treatment of alcoholism in humans , has an effect on the above described alcohol preference, flies were fed with liquid food containing naltrexone after having been fed with ethanol . The results illustrate the reversal of preference in flies pre-exposed to 15% ethanol for 48 hours and treated 0.1% Naltrexone for 24 hours. The flies not treated with naltrexone continued to show preference to ethanol food over 2h and 24h time periods, but the Naltrexone treated group showed a strong repulsion to ethanol food over 2h and 24h time points, represented by the negative preference index values in figure 6.

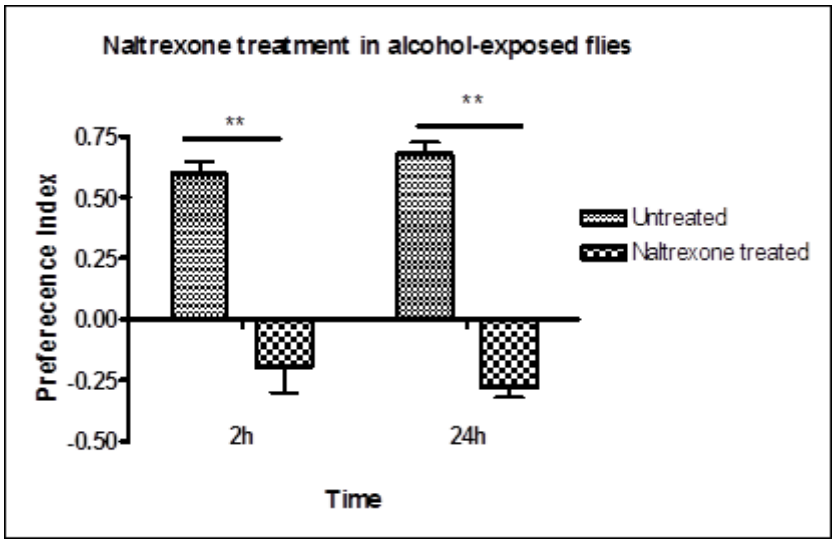

Figure 6: Effect of single dose of Naltrexone (0.1%) on alcohol preference compared to non-naltrexone treatment where all flies were pre-exposed to 15% ethanol for 48hours. Each bar represents the average of preference index of 2 experiments each with three vials for both treated and untreated groups containing 6 flies each (n=6,the number of vials). P values are calculated using students' two-tailed t-test, where $P<0.05$ indicating that there is a significant difference between the means of untreated and untreated groups. (Mean±SEM of Naltrexone treated:-0.2388±0.04117 and untreated: 0.6361±0.04073).

3.3 Naltrexone treatment in unexposed flies

In order to further investigate the reversal effect in alcohol preference shown above (Fig 6) flies were-exposed to normal food (not containing ethanol) for 48 hours and then treated with 0.1% Naltrexone. Following a 24h starvation period, preference index values were calculated using the CAFE assay. It can be observed from the figure 7, that unexposed flies showed a positive preference index values after 2h and 24h time points indicating that naltrexone did not significantly lowered/reversed the preference of flies to consume ethanol food when they had not previously been exposed to ethanol.

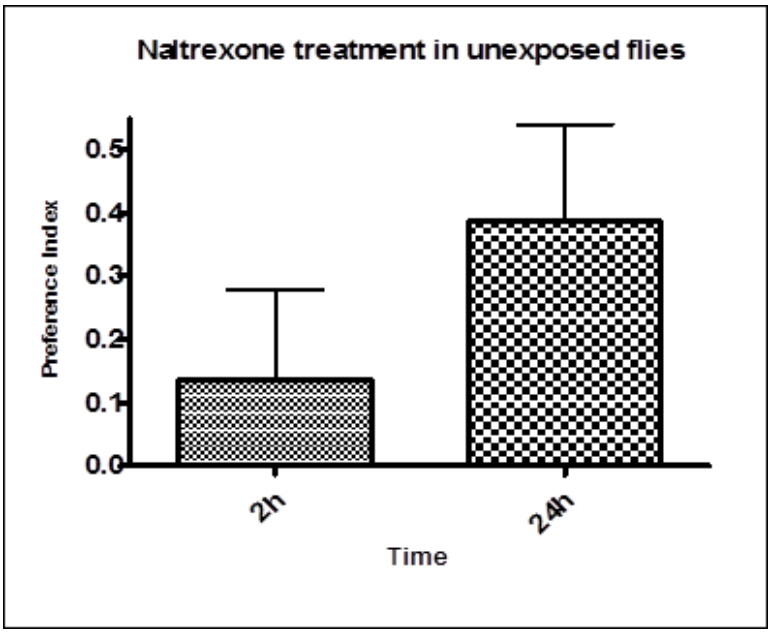

Figure 7: Naltrexone treatment in unexposed flies. Each bar represents the average of two experiments each with 3 vials for each condition (n=6, the number of vials).

3.4 Naltrexone dose-response Preference Assay

Preference assays conducted in the presence of different concentrations of naltrexone showed a dose-dependent pattern of preferential ethanol consumption over 2h and 24h time periods. In the figure below, the dot-bars indicate the preference values over 2h time and checker-bars represent 24h preference values. The 0% Naltrexone were the untreated group of flies which showed the regular pattern of preference to consume ethanol food. The reversal of ethanol preference in the presence of different doses of Naltrexone, by treatment groups of flies is represented by negative PI values in the figure below. With increase in the concentration of naltrexone treatment, an increased repulsion to consume ethanol food has been observed over 2h and 24h time points.

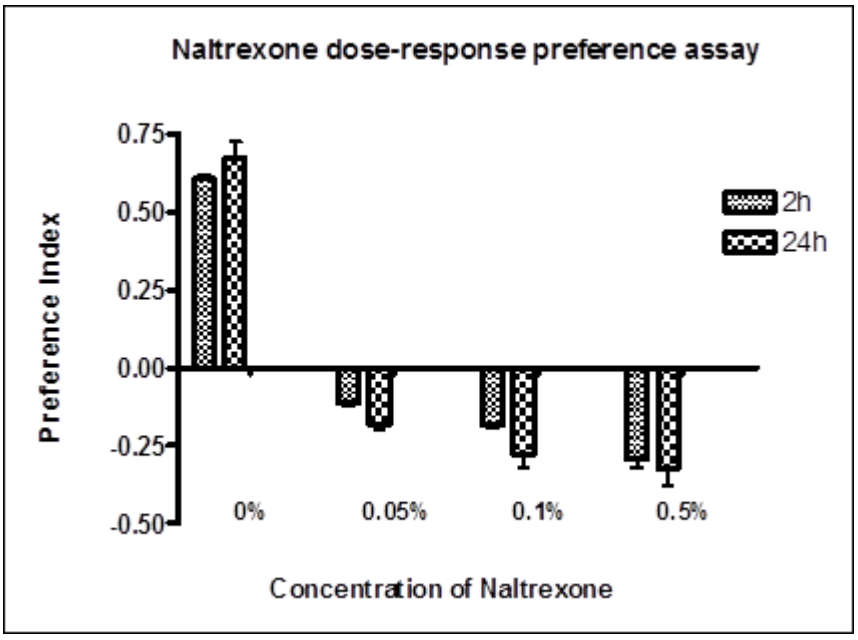

Figure 8: Dose response effect of naltrexone on alcohol preference assay in flies pre-treated with ethanol for 48hours. Statistical analysis using One-way ANOVA showed that the data is significant with $P < 0.05$. Data is the average of the preference index of 2 experiments, each with three vials for each condition (n=6, the number of vials).

3.5 Time duration of Naltrexone treatment

Having demonstrated that naltrexone is capable of reversing the preference for alcohol, it was of interest to investigate whether this effect was permanent or lasted only for the period during which the drug would still be present in the flies. Preference assays were carried out at different times after the exposure to Naltrexone in flies pre-exposed to ethanol. The results (fig 9) indicate that if the preference assay is carried out 24 hours after Naltrexone treatment there is a negative preference for alcohol (treatment 'b') while if the preference assay is carried out 48 hours after naltrexone treatment the flies display a strong preference for alcohol(treatment 'a') which is not different from flies that have not been treated with naltrexone and assayed at the same time after the last exposure to alcohol (treatment c) These results suggest the reversal effect of naltrexone is not permanent but occurs only in presence of the drug.

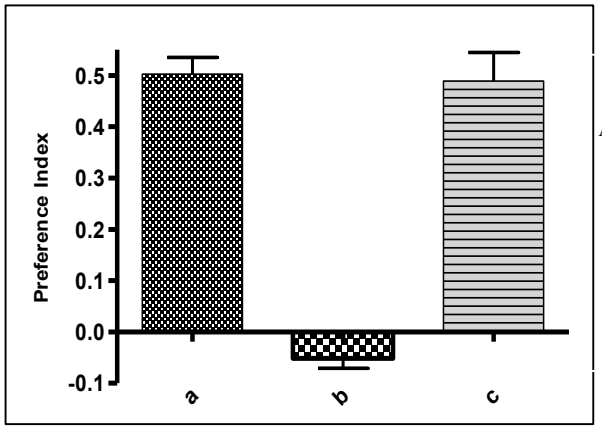

Figure 9: Preference values under different naltrexone treatment regimens as shown above. Each bar represents the average of two experiments each with 3 vials for each condition (n=6, the number of vials). Statistical analysis using One-way ANOVA showed there is a significant difference between groups a & b and b & C with $P<0.05$, but groups a & c were not statistically different with $P>0.05$.

3.6 Investigating the mechanism of action of Naltrexone-PKC kinase activity assay

In order to establish whether the Naltrexone-induced reduction of ethanol preference occurs via a pathway that is associated with alcohol addiction, it was investigated whether Naltrexone could reverse the known effect that alcohol exposure has on PKC. A PKC kinase activity assay was employed to demonstrate this effect. PKC Kinase activity was estimated using the commercially available kit as described in the methodology. In the figure 10, dot-bar represents the % activity of PKC in unexposed group of flies, checker-bar represents PKC activity expressed by ethanol (15%) exposed flies whereas striped-bar indicates Naltrexone (0.1%) treated group. It can be observed that the activity of PKC was increased in exposed group of flies compared to unexposed flies and that this activity was decreased in the presence of Naltrexone.

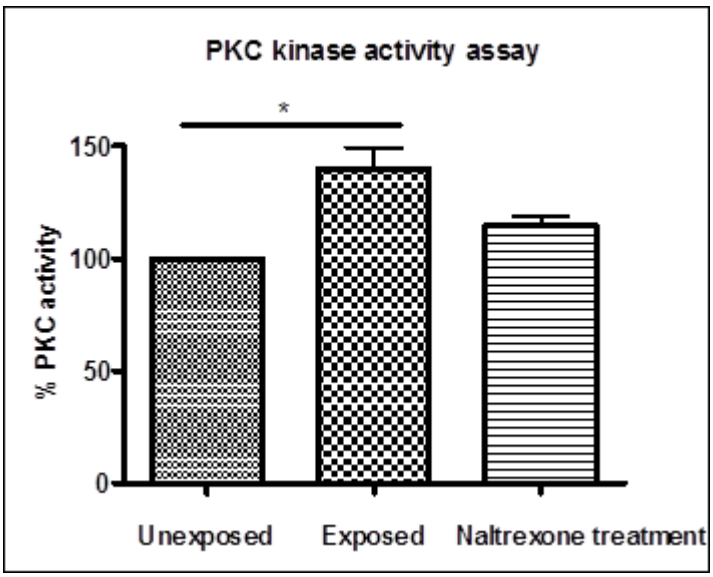

Figure 10: PKC activity in three groups of flies: Unexposed, exposed and Naltrexone treated flies. The unexposed and exposed groups are statistically different with $P<0.05$. Data is the average of 2 experiments, each carried out in triplicates (n=6).

3.7 Preference Assay in *Drosophila* mutant line (20790)

To further investigate whether Naltrexone-reversal effect is related to changes in PKC, a *Drosophila* mutant line-20790 which carries a PKC insertion-deletion of the PKC53E gene was used in alcohol behavioural experiments. Using similar preference assay conditions used for wild type flies, mutant flies were exposed or unexposed to 15% ethanol to observe preferential ethanol consumption and also under naltrexone treated and untreated conditions. Using the standard formula, preference index values were calculated and results are presented in figure 11. The unexposed group of flies have shown a little or no preference to consume ethanol food while a strong preference was displayed by exposed group of flies and this preference can be seen to be reversed by naltrexone treatment.

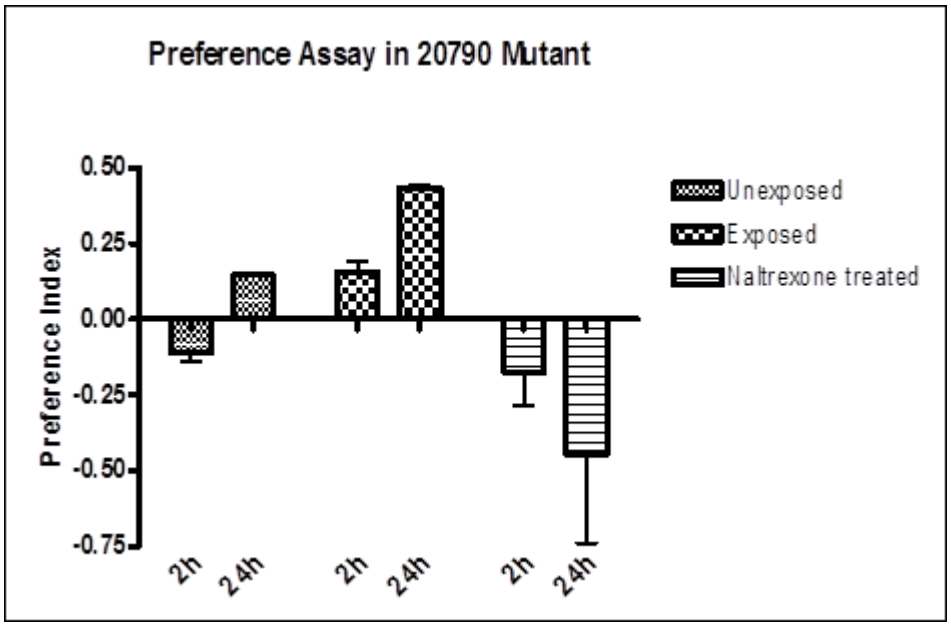

Figure 11: Preference assay conducted in mutant line (20790) of *Drosophila*. Mutant flies are unexposed or pre-exposed to 15% ethanol for 48h following a 24h starvation period. Each bar is a representative of group of 6 flies in three vials for each experimental condition (n=3).

3. DISCUSSION

The aim of this project was to further investigate the suitability of *Drosophila melanogaster* as a model for studying mechanisms of alcohol addiction and in particular to investigate whether the drug naltrexone, currently used for the clinical treatment of alcoholism, also has an effect on the flies' response to alcohol. The CAFE assay was used to measure the changes in behaviour in the flies following extended pre-exposure (48hrs) to alcohol. The results (fig 5) demonstrate that *Drosophila* prefer to consume ethanol food when given a choice between non-ethanol food and food plus ethanol but only when pre-exposed to ethanol (15% v/v). As a further development of the CAFE assay, throughout this study measurement of the alcohol consumed were carried at both 2 hours and 24 hours. The reason for this is to determine to what extent the flies make pre-conditioned decision to consume alcohol (2 hr time point) or learn/re-learn to prefer to consume alcohol (24 hr time point). Ideally one would want to use a shorter time than 2 hours for the first time point, but the consumption of food was too low and variable if measured at a shorter time interval. The results indicate that the preference for alcohol was increased in the 24 hr measurement as opposed to the 2 hr measurement. Such an increase did not occur in the non-pre-exposed flies suggesting that the 24 hr period in a choice situation is not sufficient to induce a preference for alcohol. The results are consistent with the findings of previous studies conducted on *Drosophila*'s preferential ethanol consumption (Devineni and Heberlein, 2010, Ja et al., 2007). Having confirmed the reliability of the CAFE assay, it was used to determine the effect of the opioid antagonist naltrexone on *Drosophila*'s preference for ethanol food. The results show (fig 6) that the preference of flies to consume alcohol food over normal food was

reversed by naltrexone (0.1%) treatment as indicated by the negative preference index values. This repulsion to consume alcohol food has shown to consistently increase over 24h time period. To confirm the pharmacological action of naltrexone, increasing concentrations of the antagonist were used to alter *Drosophila's alcohol* preference. It was observed (Fig 8) that the reversal of ethanol preference by naltrexone was dose-dependent over 2h and 24h time periods. This result is consistent with the idea that naltrexone is operating through a specific target which affects the flies' behaviour.

Naltrexone did not only overcome the preference for ethanol but actually induced a reverse behaviour where the flies preferentially chose non-ethanol food. To understand the reason for this response, flies that had not been pre-exposed to ethanol were treated with naltrexone and then tested in the CAFE assay (Fig 7). Naltrexone treated flies showed no preference towards ethanol consumption when measured at the 2 hr point. However, when measured at 24 hrs point there was a positive preference index which contrasts with the no preference observed in naïve flies (Fig 7). Two points can be made from these experiment. The negative preference for alcohol induced by Naltrexone is not as a result of Naltrexone alone but it is related to the 'seeking state' of the flies. Also there is the possibility that Naltrexone may have a dual action: reversing the preference for alcohol in flies that have been pre-exposed to alcohol but facilitating the induction of seeking state when Naltrexone is presented to flies not previously exposed to ethanol.

In order to further investigate if the effect of Naltrexone on reversal of preference was a permanent effect or lasted for the length of period in which the drug would be still present in the flies, preference assays were carried out at different time points after naltrexone treatment in flies that are pre-exposed to 15% ethanol. It can be observed from figure 9, that flies showed a negative preference or repulsion to ethanol in

preference assay carried out 24h after naltrexone treatment (group 'b') (which is condition similar to the one in Fig 6 and 8) whereas group 'a' showed a strong positive preference to ethanol in preference assay 48h after naltrexone treatment which is similar to the preference displayed by untreated group 'c' assayed at the same time as group 'b'. These results indicate that the effect of Naltrexone in reversal of ethanol preference is not permanent but last for a limited period presumably for as long as the drug is still present in the flies.

In order to establish that behavioural changes observed after Naltrexone consumption actually represent an alteration of the response to alcohol associated with addiction, experiments were carried out to study effects of naltrexone on Protein kinase C. PKC isozymes have been implicated in addiction processes and ethanol exposure effects their expression levels in the brain. Studies have suggested that sensitivity to alcohol in Drosophila is related to PKC isozymes and that characterizing the cellular targets of PKC may provide novel insight into mechanisms underlying alcohol addiction (Olive and Newton, 2010).

To estimate the changes expression levels of PKC in flies exposed or unexposed to ethanol and to further explore whether these changes in expression are altered in presence of naltrexone, a PKC kinase activity assay was performed using commercially available assay kit (Abcam). Three groups of flies were used for the assay- flies which are not pre-exposed to ethanol; flies pre-exposed to 15% ethanol for 48h and flies that are given Naltrexone treatment following a 48h ethanol exposure. Results of PKC kinase activity assay, (Fig 10), showed an increase in the PKC activity in the ethanol exposed flies compared to unexposed group and this activity was decreased in the presence of Naltrexone. The increase in expression levels of PKC in response to ethanol exposure is consistent with the data from previous animal studies

(Wilkie et al., 2007). However this is the first time that naltrexone has been shown to reverse the effect of alcohol on PKC activity. This results strengthen the concept that naltrexone is acting on alcohol related addictive behaviour in the flies.

In order to further investigate relationship between naltrexone changes in PKC levels, ethanol consumption experiments were carried out in *Drosophila*-PKC53E-insertion/deletion mutant (Bloomington line 20790 carrying a transposon insertion which alters the expression and function of the PKC isomer 53E). Mutant flies that were not pre-exposed to ethanol showed no preference for ethanol at 2 or 24hr (Fig 11). Mutant flies that were pre-exposed to ethanol for 48h displayed a positive preference index values indicating preferential consumption of ethanol food over normal food. This effect was reversed by Naltrexone (0.1%) The behaviour shown by the mutant flies was similar to that of the wild type flies in regards to preferential ethanol consumption and response to naltrexone treatment. These results would indicate that either the 53E isoform of PKC is not at all involved in the response to alcohol exposure, or that the ethanol-induced changes in PKC are downstream from the point of action of naltrexone and thus naltrexone has the same behavioural effect on the wild type and mutant line

4.1 Conclusion

For the first time, this work has demonstrated that naltrexone reverses the preferential ethanol consumption in *Drosophila melanogaster*. This reversal effect is consistent over time and is dose-dependent. Naltrexone also reversed the ethanol-induced increase in PKC. These data suggest that naltrexone affects a signalling pathway that is involved in the alcohol induced preference response in *Drosophila*. What remains to be stablished is the actual target of Naltrexone. Analysis of the *Drosophila* genome

does not identify any sequence with clear homology with the mammalian opioid receptors. It is however possible that another opioid binding protein exist in the flies that mediates the action of naltrexone. It would be of interest to identify such a naltrexone binding protein because it could be possible this protein is present not only in the fly but also in mammalian species and may be responsible for mediating the effect of naltrexone in mammalian species as well. Independently form the actual target of Naltrexone the results here presented allow to develop a model for alcohol seeking behaviour. It can be postulated that such behaviour is normally regulated by excitatory and inhibitory pathways. Repetitive exposure to ethanol may potentiate an opiate pathway that inhibits an inhibitory pathway regulating seeking behaviour resulting in an increase of seeking behaviour. Naltrexone would block the inhibitory action on the inhibitory pathway thus allowing inhibition of the seeking behaviour.

4.2 Future Work

The findings here described have identified a novel effect of Naltrexone on preference to consume alcohol in *Drosophila melanogaster*. Based on these data, new hypotheses can be proposed to further investigate the mechanisms underlying this reversal effect. In addition to the search for the naltrexone target above described, further pharmacological work using for example GABA, Glutamate or Dopamine antagonists could be used to to determine on which pathway the 'opiate' pathway is acting upon.

We have demonstrated that naltrexone affects ethanol induced PKC activity. This interaction could be further investigated using immunohistochemistry to further examine the expression levels of specific subtypes of PKC in specific brain regions of *Drosophila* as studies suggest that a differential expression of PKC isozymes have been observed in different brain regions.

Genetic screening methods can be used to identify novel genes that mediate/influence ethanol preference in Drosophila and possible effect of Naltrexone on those gene expressions might help understanding the underlying mechanisms. It would be of interest to identify neural circuitry that underlying alcohol preference and to further determine whether these overlap with known reward mechanisms mediated by Dopamine. The effect of Naltrexone on these mechanisms/receptors/genes can then be investigated

This work has also demonstrated the usefulness of *Drosophila* as model for alcohol addiction. Drosophila could be used a simple screening method for novel compounds that could eventually be used to treat alcoholism in humans

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