NEUROMODULATORY MECHANISM UNDERLYING ETHANOL-INDUCED BEHAVIORAL DISINHIBITION IN DROSOPHILA
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Ethanol, an active ingredient of alcohol beverages, is the most abused drug around the world and has numerous negative effects on behavior, including cognitive and motor impulsivity and addiction. The effects and underlying mechanisms of chronic ethanol exposure have been addressed in the genetically tractable *Drosophila*, as Ethanol affects behaviors of *Drosophila* and humans in a similar manner. In particular, ethanol increases disinhibited courtship between wild-type male flies (cognitive impulsivity) and the level of disinhibited courtship increases with additional ethanol exposure (behavioral sensitization). However, the cellular mechanisms underlying these phenomena are unknown. Here, we used genetic approaches to manipulate dopamine receptors and transporter to investigate their roles in the disinhibited courtship under the influence of ethanol. We also investigated the role of additional neuromodulator Amnesiac (Amn), a putative neuropeptide, since Amn is previously shown to be crucial for the ethanol’s effect on loss of motor control or sedation. Mutant flies defective in D1 receptor showed higher levels of male-male courtship compared to the wild-type flies upon exposure to ethanol. However, D2 mutant flies did not display behavioral sensitization, a type of plasticity associated with addiction. Dopamine transporter mutant *fumin*, which is unable to reuptake released dopamine and thus presumably has an enhanced dopamine level, showed drastically reduced disinhibited courtship. *Fumin* mutant flies, on the other hand, exhibited enhanced motor impulsivity. These observations indicate the critical role of dopamine in ethanol-induced cognitive and motor impulsivity. Further characterization of
the neural sites and downstream effectors that dopamine mediates ethanol-induced behavioral changes should help clarify the underlying cellular mechanisms. Various amn mutants showed inconsistent behavioral responses under chronic ethanol exposure. While amnl mutants exhibited enhanced sensitivity to the sedative effect of ethanol and enhanced intermale courtship, other amn alleles showed normal sensitivity or reduced intermale courtship. Continued studies on the molecular and cellular characteristics of different amn alleles should help clarify discrepancies in the phenotypes and provide insights into the role of Amn in the ethanol-induced sedation and cognitive impulsivity.
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To my best friend, my husband, Ali,

To my beloved parents,

And to my grand mother
Chapter 1

Introduction

1.1 Ethanol as a Drug of Abuse

Drug addiction is one of the highest medical burdens to the society. This chronic mental disorder is defined as the use of the drug despite negative consequences. Drug addiction affects normal brain functions such as learning and memory and subverts various behaviors in humans. One of the most common drugs of abuse throughout the world is ethanol. For many years people used alcohol for different purposes, from disinfection to means of partying. Ethanol is the leading cause of highway death and violence. However, our understanding of the molecular effects and mechanisms by which ethanol affects the brain and behavior is rudimentary [Wolf et al., 2003].

Most of the abused drugs such as nicotine, heroin, cocaine and heroine affect brain functions through specific target molecules. Ethanol seems to act on many targets. Previously, it has been thought that ethanol freely enters the cell membrane and alters the membrane fluidity. However, ethanol’s effects are mediated through several membrane bound proteins [Heberlein, 2000]. For example, the specific brain proteins such as receptors for γ-aminobutyric acid (GABA) type A, N-methyl-D-aspartate (NMDA) receptor, 5-hydroxytryptamine 3 (5-HT 3) receptor, voltage-gated Ca\textsuperscript{2+} channels and G-protein-activated inwardly rectifying K channels are sensitive to ethanol. However, the
mechanism of ethanol’s action on these receptors and how it relates to ethanol-induced behavior are not completely understood [Wolf et al., 2003].

Ethanol has a very simple structure composed of one OH group and the short carbon backbone. The carbon backbone group is responsible for weak hydrophobic interactions. Ethanol molecules do not make covalent or ionic bonds. All together these characteristics make ethanol a very potent drug that brain concentrations of approximately 5 to 10 mM could result in intoxication and anesthesia. As a result of low potency of ethanol, pharmacological assays like radioligands can not be used to establish the site of ethanol binding and interactions with target proteins. Moreover, distribution of ethanol in the cell and its weak reactions with proteins are some of the obstacles researchers have encountered working on ethanol [Lovinger et al., 2005].

Low dose ethanol consumption in humans induces euphoria and disinhibition. When ethanol is used at high doses, it will cause confusion and incoordination of movements. Intoxication with extreme levels of ethanol can lead to coma and even death in some cases. Genetic predisposition has been shown to play an important role in the degree of response to ethanol in human. Persons without a family history of alcoholism have higher sensitivity towards the perceptual, motor and even biochemical effects of ethanol intoxication compared to ones with the family history of alcoholism. Additionally after decades of ethanol abuse, people with higher ethanol sensitivity had lower level of alcoholism. Taken together, genetic background has a great influence on the individual’s response to ethanol consumption and in part could predict the risk of ethanol addiction [Heberlein, 2000].
Chronic ethanol consumption in human leads to tolerance development. Tolerance is defined as the resistance acquired towards the effects of ethanol [Scholz et al. 2000]. In humans, tolerance develops more quickly to aversive effects of alcohol compared to its pleasurable effects. The initial tolerance towards the aversive effects of ethanol has been proposed to be the basis of dependence and addiction to ethanol [Tabakoff et al, 1988].

Ethanol has somewhat similar effects in rodents as in humans. Ethanol in rodents first increases the locomotor activity and then gradually induces sedation. Toxic effects of ethanol also could lead to death of the animal. Rodents, like humans, develop tolerance to the sedative effects of ethanol. Rapid tolerance is induced after a short-term exposure of the animal to ethanol and chronic tolerance is observed after repeated exposure to ethanol. Alteration in disposition of ethanol, such as absorption, metabolism and excretion are proposed to be the bases of developing tolerance. However, ethanol tolerance is also induced at physiological or pharmacodynamic changes in cellular functions. Adaptive changes of neurons in the brain to the effects of ethanol that resemble learning and memory processes could be in part responsible for developing tolerance[Scholz et al. 2000]. Moreover, applying neuropeptide and neurotrophins, which affects neuroplasticity in mice has been shown to both increase acquisition and decrease disappearance of tolerance. Observation on selective breeding in mice has proposed that genetic factors also play an important role in developing tolerance to ethanol [Crabbe et al, 1979].

*Drosophila* also rapidly develops tolerance even after single intoxicating ethanol exposure. Flies become resistant to the effects of alcohol on motor incoordination and sedation. Development of tolerance in fruit fly is not due to changes in absorption and
metabolism of ethanol. Rather, disruption of structure and function of the central complex in the *Drosophila* brain by genetic manipulation causes reduction in acquisition of tolerance [Scholz et al. 2000]. Thus, adaptation of neuronal activity to ethanol has been suggested to be the basis of developing tolerance in *Drosophila*. Furthermore, octopamine, one of the major monoamines in insects, is involved in developing tolerance to the sedative effect of ethanol [Scholz et al. 2000].

### 1.2 *Drosophila* as a Model to Study Ethanol’s Effects

For many years *Drosophila melanogaster* has been used as a model organism to study drug addiction and specially ethanol addiction. During evolution, the genes and biochemical pathways underlying behavior and development has been highly conserved from *Drosophila* to mammals. Thus, many genes characterized in *Drosophila* have led to a better understanding of similar molecular functions for other vertebrate and even human behaviors [Heberlein, 2000]. *Drosophila* has approximately 200,000 neurons in the central nervous system (CNS), which is simpler than the mammalian CNS yet mediates complex behavior. For example, *Drosophila* is capable of forming and retaining associative memory of many environmental cues [Kim YC at al, 2006]. They also show a sophisticated courtship behavior [Heberlein, 2000].

There are many additional advantages of using *Drosophila* as a model to study behavior. *Drosophila* is inexpensive and easy to rear, and has a relatively short generation time, approximately 10 days at 25°C, which has enabled researchers to monitor and document the heritability of certain behaviors [Heberlein, 2000].
Furthermore, it has been suggested that the evolutionary origin of human ethanol ingestion might be related to primate frugivory, which has been led to high amounts of alcohol consumption in humans [Dudley, 2000]. Thus, *Drosophila melanogaster* and human have nearly the same history of alcohol exposure [Heberlein et al., 2000].

Availability and simplicity of genetic manipulation in *Drosophila* is a major advantage of using flies as a model organism [Heberlein et al., 2000]. In addition, complete sequence of the *Drosophila* genome has been identified. The functional similarities between *Drosophila* and mammalian genes are another important advantage of using *Drosophila*. Previous research in *Drosophila* has generated many genetic tools, such as deficiency lines with small deleted chromosomes, which can be used to map novel mutations, transposable element-mediated mutation, gene tagging and germ-line transformation and targeted gene disruption. Those tools have facilitated further genetic studies in *Drosophila* [Heberlein, 2000].

*Drosophila* has been used to study CNS development and functions. As in the mammalian CNS, the *Drosophila* CNS has serotonin, dopamine, gamma amino butyric acid (GABA) and glutamate, the potential targets of ethanol. Therefore, *Drosophila* could be one of the best available animal models to investigate the effects of ethanol on both behavior and nervous system functions [Heberlein, 2000].

One of the approaches to identify the cellular and molecular mechanisms underlying ethanol’s effects and addiction is to investigate the changes that occur after prolonged alcohol exposure. Neuronal adaptations that lead to tolerance, dependence and withdrawal symptoms are caused by chronic alcohol consumption [Lovinger et al., 2005]. In order to identify the molecules mediating ethanol-induced behavior, flies
treated with ethyl methane sulfonate (a chemical which induce DNA lesions) have been exposed to ethanol to select the mutant strains with altered sensitivity to ethanol intoxication [Singh et al, 2000]. Numerous *Drosophila* mutants with increased or reduced ethanol sensitivity have been identified. For example, *tipsy* flies showed increased sensitivity to ethanol while *barfly* showed decreased sensitivity to the sedative effect of ethanol. However, both *barfly* and *tipsy* flies responded normally after exposure to ethanol. These studies showed that the genes that control the locomotor activating effect of ethanol are different from the genes that mediate the sedative effects of alcohol. Identification of these genes could lead to better understanding of mechanisms by which ethanol alters behavior [Heberlein, 2000]. Moreover, studies performed in rodents also show similar results to *Drosophila* [Shen et al, 1996].

Another approach to generate *Drosophila* mutants with altered ethanol sensitivity is to use P-elements [Engels et al, ]. P-elements are transposable elements that could be somewhat randomly integrated into *Drosophila*’s genome and inactivate the genes located near their insertion sites. One of the mutants generated by the P-element-mediated mutagenesis is *cheapdate/amnesiac*. The *cheapdate* flies carry a mutation in amnesiac gene, which is involved with learning and memory [Moore et al.,1998]. The mammalian homolog of amnesiac gene is believed to be a neuropeptide PACAP [pituitary adenylyl cyclase (AC) activating protein]. AC makes cAMP, which activates a signal transduction pathway crucial for many cellular functions [Heberlein, 2000].

Studies of *cheapdate* and other mutants have shown the role of the cAMP signaling pathway in mediating ethanol’s effect on sedation [Moore et al.,1998]. The *Drosophila* mutants with impaired cAMP-regulated protein kinase A (PKA-RII) has been
shown to be resistant to the ethanol’s sedative effect compared to wild type flies [Li et al, 1995]. Mutant mice defective in the homologous gene PKA-RII are also more resistant to the sedative effect of ethanol [Thiele et al, 2000]. Thus, disruption in the cAMP signaling pathway affects sensitivity to ethanol. Interestingly, studies on human subjects reveal that alcoholics have decreased level of AC activity [Tabakoff et al, 1988], suggesting that the cAMP signaling pathway is crucial for the ethanol’s effect on sedation. Therefore, the similar results made in the *Drosophila*, mouse and human studies on PKA-RII provide strong evidence on the evolutionary conservation of the molecules mediating ethanol’s effects [Heberlein et al., 2000]. Moreover, quantitative measurements of ethanol tolerance such as maximal tolerance level and kinetics of tolerance disappearance are similar between flies and rodents [Scholz et al. 2000]. Taken together, *Drosophila* is a suitable model to study the effects of ethanol.

Nonetheless, there have been some concerns on using invertebrates as a model for alcohol study. Considering the simplicity of the fly brain and behavior, there may be difficulties to investigate certain psychological effects of ethanol such as anxiety, impulsivity, craving and increased sensitivity to rewards [Lovinger et al., 2005]. Other features such as social pressure referred to as peer pressure would never be modeled in non human models. The findings described in this thesis at least demonstrate that *Drosophila* is an excellent model to study the effect of ethanol on impulsivity.
1.3 Behavioral Assays of Ethanol Intoxication in Flies

1.3.1 Locomotor Behavior

*Drosophila melanogaster* is a fruit fly that naturally lives on fermenting foods and environment. Fruit flies use ethanol as a food source and also as a precursor for lipid biosynthesis [Heberlein et al., 2000]. Ethanol intake as a food source during their life makes flies to be resistant to the effects of ethanol. Researchers have added ethanol to *Drosophila*’s food or have exposed the flies to ethanol vapor to identify different strains of *Drosophila* with enhanced or decreased resistance to ethanol [Geer et al, 1993]. Furthermore, researchers have generated *Drosophila* strains with increased resistance to ethanol in the laboratory by selectively breeding the ones that survived higher amounts of ethanol in food [Heberlein, 2000].

Many aspects of fly behavior would change upon exposure to ethanol. Locomotor behavior is one of these behaviors that have been studied for many years. Upon exposure to ethanol, *Drosophila* shows a complex behavior; at the beginning of ethanol exposure, locomotor activity of the flies will increase but soon after, it will change its position to incordination, loss of postural control, eventually sedation and immobility [Wolf et al., 2003]. Flies under the influence of ethanol would also change their walking behavior; Low doses of ethanol increase the walking speed and eventually cause loss of motor control and sedation. Intoxicated flies would also change their walking directions more often than ethanol-naïve flies. Changes in walking speed have been quantified using a simple line crossing assay, an automated beam braking assay and a video tracking system [Rothenfluh A. et al.,2002].
Another way to assess the effects of ethanol on *Drosophila* is to measure loss of postural control by using an inebriometer. The inebriometer is a device to assess the effects of ethanol on fly postural control or sedation. The inebriometer is a vertical cylinder with series of oblique mesh that allows the flies to rest and walk. Different concentrations of ethanol vapors could be induced inside the inebriometer and the behavior of flies could be monitored afterward. The flies remain near the top of cylinder because of their natural inclination for negative geotaxis. Eventually flies get intoxicated, lose their postural control and then eventually become sedated and fall to the bottom of the inebriometer. Mean elution time (MET) of the flies can be calculated for quantitative analysis. Since the level of ethanol absorbed in flies is linear with time, the MET is use to measure sensitivity of flies to the sedative effect of ethanol [Wolf et al., 2003]

1.3.2 Behavioral Disinhibition

The most important role of the male sex in nature is to mate with the female. Male sexual behavior consists of not only copulation but also behavioral steps preceding copulation. These behavioral steps would let the male locate the mate, assess the suitability of the mate and also stimulate a receptive response in the partner. In almost all species, appropriate and successful execution of these behaviors is the essential means for survival and reproduction. Male sexual behavior in *Drosophila* starts with orienting and then following. First, the male will orient himself to the female and then he begins to follow the female in the direction she moves. After that, tapping takes place in which the
male will touch the female with its forelegs. The next actions will be singing, where the male vibrates his wings, and then licking the female’s genitalia. Finally, the male curls its tail and copulates with the female. Visual, olfactory and gustatory cues play very important roles in each step of the sexual behavior [Emmons et al., 2003].

Typically a male fly will court a female that has attractive pheromones. Rarely, a male fly may attempt to court another male, which will quickly separate and move away. However, upon chronic exposure to ethanol, male flies show a distinct behavior of courting other males, which is called disinhibited courtship. Male flies under the influence of chronic ethanol actively court each other along with female flies as well [Lee et al., 2008]. The male flies that have been exposed to ethanol can make pairs and also chains of courting males, which usually lasts for 5 to 10 minutes before they become sedated. This unique behavior has been only observed in males while female flies upon exposure to ethanol do not court each other presumably because their sexual behavior is typically passive [Lee et al., 2008]. While dopamine is shown to be crucial for the ethanol-induced disinhibited courtship [Lee et al., 2008], the underlying cellular mechanism is unknown and thus needs to be investigated.

1.4 Dopaminergic System

Dopamine is one of the biologic monoamines and is involved in neuronal and behavioral plasticity in both vertebrates and invertebrates [Wolf et al., 2003]. In Drosophila, dopamine acts as a key neuromodulator of aversive and appetitive memory formation in olfactory conditioning[Schwaerzel et al, 2003],[Kim YC at al, 2006].
Dopamine appears to be crucial for sexual motivation and arousal in human, rodent and flies [Hull EM et al, 2004].

The molecular structure of dopamine consists of a benzene ring with two hydroxyl groups, a single amine group and a side chain of ethylamine [Kandel E. et al, 2000] (Figure 1.1) Dopamine is produced from the essential amino acid tyrosine. Tyrosine hydroxylase is the first and rate-limiting enzyme in dopamine biosynthesis that converts tyrosine to L-dihydroxyphenylalanine (L-Dopa). Dopa Decarboxylase is the second enzyme, which converts L-Dopa to dopamine. – need a section how dopamine is cleared (DAT) and broken down (monoamine oxidase or N-methylation).

Figure 1.1:  Structure of Dopamine

Dopamine receptors belong to G-protein coupled receptors and have been divided into two distinct subfamilies D1 and D2 receptors based on biochemical and pharmacological characteristics. D1 and D5 belong to D1 receptors while D2, D3 and D4 belong to D2 receptors and individual members of subfamilies have distinct pharmacological profile and expression patterns in the brain [Vallone et al., 2000].
Dopamine’s roles in movement control have been established previously. Pathological conditions such as Parkinson’s disease that have a decreased level of dopamine as a result of degeneration of dopaminergic neurons manifest movement abnormality. The role of dopamine in locomotor activity has been also demonstrated using D1 receptor agonists and antagonists. It has been shown that D1 receptor agonists, SKF38393 would increase motor activity, while D1 receptor antagonists as as SCH23390 or SKF83566 would decrease motor function in rodents [Vallone et al., 2000].

Dopamine plays an important role in arousal and courtship in *Drosophila*. Enhanced courtship level has been documented in the transgenic flies overexpressing vesicular monoamine transporter or tyrosine hydroxylase in dopamine neurons [Chang et al., 2006]. The study recently reported by Lee et al., shows that dopamine plays an important role in disinhibited sexual behavior of the male flies under the influence of ethanol [Lee et al.,2008]. They have shown that the level of disinhibited courtship of the male flies with inhibited dopamine neuronal activity is drastically reduced compared to the flies with normal dopamine activity. The *Drosophila* D1 receptor dDA1 is highly expressed in the mushroom bodies, the brain structure crucial for learning and memory, especially gamma lobes. In addition, the Fru^M^ mutants with altered Fru^M^ (a male sex determination factor) activity do not show ethanol-induced courtship disinhibition. Since Fru^M^ is abundantly expressed in the gamma lobes of the mushroom bodies, it is possible that the binding of dopamine to dDA1 receptors in the mushroom body gamma lobes may be critical for courtship disinhibition [Lee et al.,2008].

Ethanol is a positive reinforcer and increases dopamine release and functions in the mesolimbic reward pathway in mammals. For example, microinjection of ethanol into
the ventral tegmental area (VTA) has been shown to increase dopamine release in the nucleus accumbens (NAc). Deficiency of D1 or D2 receptor or regulatory phosphoproteins of these receptors suppresses ethanol intake and reduces ethanol conditioned place preference in mice [Weiss et al., 2002].

Although acute ethanol exposure activates and increases the dopamine level in VTA, chronic alcohol exposure induces adaptation in mesolimbic dopamine function, causing hypoactivity of dopamine neurons in VTA and NAc. Low dopamine levels in the mesolimbic system may increase desire to consume alcohol and it has been suggested the basis for maintenance of alcohol addiction. Hence, resumption of alcohol drinking may increase the dopamine level and reverse the deficit [Weiss et al., 2002].

Mechanisms of dopamine dysregulation in chronic ethanol exposure or alcohol withdrawal have been investigated by numerous studies. L-type calcium channels may play a role in this matter. Researchers have shown that hyperactivity of L-type calcium channels might be the underlying mechanism of decrements of dopamine content in the mesolimbic system during ethanol withdrawal. Moreover, by blocking these L-type calcium channels they were able to eliminate the ethanol withdrawal effects in rats [Rossetti ZL et al, 1999].

There has been some evidence that the level of tyrosine hydroxylase decreases during chronic ethanol exposure while an enhanced level of dopamine transporter (DAT) is detectable in mesolimbic system of the rats [Rothblat et al, 2001]. Hence, hypofunction of dopamine during ethanol withdrawal could be due to either reduced dopamine synthesis or increased dopamine clearance from the synapse or even both [Weiss et al., 2002].
Dopamine deficiency in VTA is not only observed in acute withdrawal but also in long lasting withdrawal, such as 3 days after deprivation of ethanol in rats [Diana et al, 1996]. Moreover, it has been shown that decrease in the dopamine level in NAc is observed even 2 months after ethanol withdrawal [Bailey et al, 2000]. The slow recovery of the level of dopamine is thought to be responsible for the relapse and poor treatment outcome in patients trying to quit drinking alcohol [Weiss et al., 2002].

Another brain structure implicated in ethanol addiction is the extended amygdala. The group of neurons extends from NAc and bed nucleus of stria terminalis (BNST) to central amygdala (CeA). Ethanol increases dopamine level in CeA and BNST. There is some evidence that extended amygdala is involved in negative reinforcement by ethanol. Anxiety and other affective changes during ethanol withdrawal have been contributed to neuroadaptive changes in the CeA and BNST. Ethanol withdrawal increases the Corticotropin Releasing Factor (CRF) secretion from CeA neurons. Since, Intracranial CRF antagonists or deletion of CRF gene reduces anxiety during ethanol withdrawal; CRF has been implicated in affective behavior induced by ethanol deprivation [Weiss et al., 2002].

In *Drosophila*, flies with pharmacologically induced dopamine reduction acquire tolerance normally compare to wild type flies. However, these flies with decreased dopamine levels show reduced locomotor stimulation under ethanol exposure [Scholz et al. 2000]. This suggests the crucial role of dopamine in the locomotor stimulating effect, but not tolerance, to ethanol in *Drosophila*.

Courtship is another behavior that has been shown to be affected by the dopaminergic system. In mammals, dopamine and serotonin (5-HT) have regulatory
effects on male courtship behavior. Pharmacological studies have shown that increasing dopamine, facilitates courtship whereas 5-HT decreases the sexual behavior in mammals [Hull EM et al, 2004]. Dopamine affects various systems to regulate sexual behavior. In *Drosophila*, dopamine modulates visual perception, male arousal and female sexual receptivity during heterosexual courtship [Liu et al., 2008]. Moreover, dopamine is important for courtship conditioning and ethanol-induced disinhibition [Lee et al., 2008].

The study by Liu et al in 2008 [Liu et al., 2008] reveals that increased dopamine level in *Drosophila* by pharmacological or genetic manipulation, increases the propensity of male flies to court other males. They used genetically modified flies that overproduce tyrosine hydroxylase that leads to increased production of dopamine. In this study, alteration in the dopamine level does not change general olfactory and gustatory responses and locomotor activity of the flies. Thus, the higher level of inter male courtship in the flies with increased level of dopamine is likely due to alteration in sensory perception of male flies of each other [Liu et al., 2008].

Interestingly in another study by Liu et al in 2009, the decreased level of dopamine enhanced the level of inter male courtship in *Drosophila* [Liu et al., 2009]. They suggest that the enhanced level of inter male courtship is related to increased attractiveness of male flies with reduced dopamine to other males. The propensity in courting other males was not enhanced in male flies with reduced dopamine. The authors suggest that a change in chemical signals in dopamine deprived flies is responsible for the increased attractiveness to other males. The study, however, has failed to identify a specific pheromonal compound responsible for attractiveness of male flies to other males. Moreover, it is possible that reduction in dopamine level may cause reduction in rejection.
efforts, leading to enhanced inter male courtship in flies with reduced dopamine [Liu et al., 2009].

Dopamine transporter (DAT) is a membrane bound protein that transports dopamine from extracellular space into presynaptic dopamine neurons. Thus, this protein plays a key role in recycling dopamine and regulating dopamine neurotransmission. DAT has been used for many years as a target for many pharmacological interventions. Many of psychostimulant drugs work on this transporter to elevate mood and increase locomotor activity [Uhl et al., 2003].

To investigate the role of DAT in *Drosophila*, Porzgen et al. have identified the mutant named *fumin* (*fmn*) that has genetic lesion in the DAT gene [Porzgen et al, 2001] Fumin in Japanese means sleepless. The behavioral phenotype of the *fmn* flies consists of increase in active waking phase and dramatic decrease in their sleep or inactive phase. Increase in the level of dopamine in the synaptic cleft is thought to be responsible for hyperactivity and restless behavior of *fmn* flies. In addition, *fmn* mutants have enhanced arousal to a mechanical stimulation, supporting the findings in mammals [Kume et al., 2005].

### 1.5 Amn and PACAP

Arimura’s group in 1989 identified pituitary adenylate cyclase-activating polypeptide (PACAP), a hypothalamic neuropeptide protein in the anterior pituitary gland [Miyata A. et al, 1989]. PACAP has 68% similarity of amino acids with vasoactive intestinal polypeptide (VIP) and 37% identity with secretin. This similarity between
PACAP and VIP/Secretin indicates that this neuropeptide is a member of VIP/glucagon/Growth hormone releasing hormone (GHRH) superfamily. PACAP is found in the CNS as well as peripheral tissues such as testes, adrenal medulla, pancreatic ganglia and the enteric nervous system. PACAP is a neuromodulator or neurotransmitter in both central and peripheral nervous system [Hashimoto et al., 2002].

Quinn et al. in 1979 isolated the first amnesiac mutant in their search for memory mutants in Drosophila [Quinn et al., 1979]. The amnesiac flies are normal in acquiring an operant avoidance memory and also retain long-term memory but defective in memory retention in the first few hours after training [Hashimoto et al., 2002]. Feanny and Quinn isolated the amnesiac cDNA and characterized genomic structure; however, the final product of amnesiac gene is yet unidentified. It has been suggested that Amn encodes several neuropeptides [Quinn et al., 1979]. Two of the potential neuropeptides are homologous to the vertebrate PACAP and GHRH. One peptide has certain sequence identity with GHRH and PACAP related peptide (PRP). PRP is derived from preproPACAP and has some homology to GHRH. The second predicted product of amnesiac gene is thought to have homology with PACAP38. It has been shown that PACAP38 activates potassium currents at the neuromuscular junction in Drosophila via AC and RAS/Raf. Since the PACAP38 immunoreactivity is found in Drosophila nervous system, it is suggested that the amnesiac products may also act through AC and cAMP [Hashimoto et al., 2002].

rutabaga and DCO are Drosophila mutants that have loss of function in the cAMP signal transduction cascade. rutabaga mutants defective in AC and DCO defective in the catalytic subunit of PKA have show increased sensitivity to ethanol [Hashimoto et
Furthermore, the study by Moore et al in 1998 reveal that *amnesiac* mutant allele *amn¹* exhibits increased sensitivity to ethanol. They have also identified another *amnesiac* mutant allelic *cheapdate* with increased sensitivity to ethanol [Moore et al., 1998].

The structure of PACAP38 is well conserved among all mammals, lower vertebrates and protectorates. However, the primary structure of GHRH is not conserved even between different mammalian species. In mammals, there are two distinct genes that produce PACAP38 and GHRH but in non-mammalian species including catfish, salmon, frog and chicken, PACAP and GHRH-like peptide are encoded by one gene. Therefore, it seems that after divergence of birds and mammals, GHRH/PACAP gene is duplicated to two distinct GHRH and PACAP genes. Since *Drosophila* belongs to non-mammalian species, it is possible that *Drosophila* amnesiac gene may encode both a PACAP-like and a GHRH-like peptide [Hashimoto et al., 2002].

Most proteins involved to olfactory learning and memory in *Drosophila* are highly enriched in the mushroom body neurons. However, the amnesiac protein is expressed mostly in Dorsal Paired medial (DPM) neurons that innervate the mushroom body axons [Tamura et al, 2003]. It has been shown that congenital ablation of neurotransmitter release from DPM neurons resembles the amnesiac phenotype [Tamura et al, 2003]. Therefore, DPM neurons have a crucial role in memory storage and thus it is suggested that amnesiac in DPM plays an important role in memory storage. Genetic rescue of amnesiac during development reverses the memory defect. If the restored Amn expression is induced only during the adult stage, the memory function is not rescued completely [Waddell et al, 2000]. Indeed, there is a developmental defect in *amnesiac*
mutants and thus Amn has an important role in the DPM neuronal development. Notably, the *amnesiac’s* increased sensitivity phenotype to ethanol is rescued by amnesiac gene expression in the adult stage. Therefore, Amn’s functions in ethanol sensitivity and memory storage have different spatio-temporal requirements [Hashimoto et al., 2002].

The cAMP signal transduction pathway plays an important role in acute and chronic ethanol exposure. Although acute exposure to ethanol increases the amount of cAMP, the chronic exposure has the opposite effect by decreasing the level of cAMP [Bellen et al., 1998]. It also has been suggested that binding of *amnesiac* to its receptor of unknown nature may increase the level of cAMP [Bellen et al., 1998]. The role of amnesiac/cheapdate in ethanol sensitivity have been previously investigated by Moore et al in 1998 [Moore et al., 1998]. In this thesis I have investigated the role of the amnesiac gene in ethanol-induced disinhibited courtship behavior in *Drosophila melanogaster*.

**1.6 Thesis Summary**

The goal of my research is to understand the molecules and mechanism underlying ethanol-induced courtship disinhibition, which represents cognitive impulsivity. Previous studies have identified the role of three cellular components FruM, White and dopamine in disinhibited courtship in *Drosophila* under the influence of ethanol [Lee et al., 2008]. To further elucidate the mechanism by which dopamine mediates courtship disinhibition, I examined the transgenic flies with either increased or
decreased neurotransmission, and genetic mutants defective in D1, D2 receptors and Dopamine transporter and the findings are documented in this thesis.

Another gene that has been shown to mediate effects of ethanol is amnesiac [Moore et al., 1998]. To better understand the molecular and cellular basis of ethanol-induced courtship disinhibition, I expanded my research to various amnesiac mutants. I hypothesized that under chronic ethanol exposure, the amnesiac flies would show higher disinhibited courtship compared to the control flies. I also rescued the amnesiac’s phenotype to confirm the role of this gene, as opposed to other factors, in ethanol-induced courtship disinhibition and to map the site of the Amn’s function.

The enhanced understanding of the mechanisms underlying courtship disinhibition in Drosophila has several potential benefits. First, identifying the genes involved in disinhibited courtship would provide a baseline to study similar phenomena in other organisms including humans. The mammalian homologs of Drosophila genes may serve as a potential candidate for intervention of cognitive impulsivity. Second, studies in Drosophila could provide better insight into the mechanism underlying similar phenomena such as disinhibited sexual behavior and aggression in mammals. Furthermore, identifying genes associated with this disinhibited courtship behavior or other alcohol related behaviors could provide clues for the development of therapy or cure in humans. Performing these studies in rodents and humans could be difficult because of the complexity of the mammalian or human systems. Therefore, by studying a simpler model system such as Drosophila, it should be feasible to identify the mechanisms by which ethanol acts on the CNS to exert negative effects.
Chapter 2

Role of Dopaminergic System in Ethanol-Induced Courtship Disinhibition

2.1 Introduction

Dopamine is a major neuromodulator regulating various aspects of mammalians and Drosophila activities. Dopamine regulates sleep and awake cycle [Kume et al., 2005], locomotor activity [Vallone et al., 2000], sexual behavior [Liu et al., 2008],[Liu et al., 2009], rewarding and disinhibited courtship under the influence of ethanol [Lee et al., 2008]. The role of dopamine in disinhibited courtship has been previously documented using transgenic flies with inhibited dopamine neurotransmission [Lee et al., 2008]. Ethanol increases dopamine level in different areas of the brain and changes in dopamine receptor and dopamine transporter have been associated with alcoholism in mammals and rodents [Vallone et al., 2000].

Dopamine needs to bind to its membrane receptors in order to exert its actions. Dopamine receptor has 5 subtypes in mammals, D1 to D5. However, there are only D1 (dDA1), D2 (dD2R) and D5 (DAMB) present in Drosophila melanogaster [Vallone et al., 2000]. Here we investigated the role of individual dopamine receptors in Drosophila disinhibited courtship induced by chronic exposure to ethanol. We also used dopamine transporter mutant, fumin flies to evaluate the effects of ethanol in flies with abnormal dopamine activity.
2.2 Material and methods

2.2.1 Fly Stocks

We used Canton S (CS) from Bloomington center, as a wild type strain. The \textit{dumb}^1(1176) and \textit{dumb}^2 flies, which are both impaired in the D1 receptor dDA1, were previously characterized in Dr. Han’s lab by Youngcho Kim [Kim YC at al, 2006]. D2 mutant flies which are mutant in D2 receptor were obtained from the Bloomington Stock center. \textit{Elav-GAL4} and \textit{tubp-GAL80ts} flies were previously provided by M. Heisenberg and Dr. Davis, respectively. Moreover, \textit{fumin} flies (DAT mutant flies) were kindly provided by Dr. Jackson.

2.2.2 Fly Culture

We used the standard cornmeal agar medium to rear flies at 25° C. The flies were kept at 12 h light/12 h dark photoperiod with 50% relative humidity. Male flies were used for the behavioral assay. Flies were collected within one to two days after eclosion under CO2 anesthesia and transferred to food vials. We used 33 males as a group and aged them for 4-5 days after eclosion for the behavioral assay. Each experiment of ethanol exposure was carried out on 6 groups of 33 males of various genotypes under test. In order to normalize environmental conditions, we tested equal numbers of control and mutant groups.
2.2.3 Behavioral Assays

Each group of 33 male flies was exposed to ethanol in a novel apparatus called Flypub at room temperature (23°C). Flypub (Figure 2.1) is a plastic, cone shaped chamber that is 57 mm in diameter on top and 103 mm in height (57mmD x 103 mm H). Flypub has a clear ceiling for videotaping fly courtship behavior. Flypub has an open bottom with the diameter of 40 mm for ethanol administration. A plastic mesh was inserted at 2 cm down from the top of the Flypub. This mesh provides a place for flies to rest and walk. There is a small hole on the side, above the mesh, in order to introduce the flies to the Flypub with a small funnel.

Figure 2.1: Schematic presentation of Flypub
Flies were transferred to the Flypub very gently to minimize stress and were acclimated to the chamber for 10 min. One ml of 95% or 70% ethanol was applied to a small cotton pad placed in a Petri dish (35mm D), which was covered with Kimwipes to allow slow diffusion of ethanol vapor. Flypub was placed on the ethanol-containing petri dish and videotaped using a Sony HAD CCD camera (Avalonics, NY, USA) till all flies were sedated. The sedated flies were transferred back to food vials and kept in the incubator until the next ethanol exposure. Flies were exposed to ethanol once a day for 6 consecutive days.

During ethanol exposure, the number of sedated flies was counted every 3 minutes to measure sedation time. The flies lying on their back or not moving for more than 10 seconds were scored to be sedated. Mean Sedation Time (MST) was used to compare the sedative effect of ethanol in different strains. MST was obtained by multiplying the number of sedated flies at each time point by the time of sedation, adding the values in all time points and then dividing the total value by the total number of flies in each Flypub.

Courtship activity was monitored in the recorded videos of the flies on the first, second, third and sixth ethanol exposure. The maximum number of flies engaged in courtship was scored in each 30 second interval. We used the average of 10 consecutive periods to represent the percentage of males engaged in courtship. The sporadic courtship activity that lasted only for a couple of seconds was excluded. All experiments were blinded to the experimenters for both ethanol administration and scoring.

Statistical analysis was performed using Minitab 15 (Minitab Inc., State College, PA, USA). All data are reported as mean ± standard error of the mean. Two-tailed student
t-test was used to compare the means of two groups. In case there were more than two groups to compare, we performed analysis of variance (ANOVA).

2.3 Role of The Dopaminergic System in Tolerance Development to the Sedative Effect of Ethanol

To investigate the chronic effects of ethanol on behaviors of *Drosophila*, we exposed the flies to ethanol in Flypub with a 95% or 70% ethanol-containing pad. Upon exposure to ethanol, flies started to move faster, indicating that they became hyperactive. Eventually they lost their postural control, fell frequently to the mesh in Flypub and finally became sedated as judged by the observation that they laid on their back.

In order to investigate the role of the dopaminergic system in the sedative effect of ethanol and tolerance development, I tested *dumb*¹, *dumb*² and D2 receptor mutant flies along with CS as a control. MST of *dumb*¹ and *dumb*² on the first exposure to 95% ethanol were 19.10 ± 0.28 min and 20.63 ± 0.51 min, respectively, which were not significantly different from that of CS, which was 20.05 ± 0.30 min (N=6, p > 0.05; Figure 2.2 ). On the second 95% ethanol exposure, *dumb*¹ and *dumb*² had MST of 23.97 ± 0.50 min and 25.31 ± 0.77 min, where CS male flies had the MST of 23.27 ± 0.58 min (N=6, no significant difference among *dumb*¹, *dumb*² and CS, p > 0.05). Similar to CS flies, *dumb*¹ and *dumb*² flies developed tolerance to the sedative effect of ethanol exposure that did not change significantly during the following ethanol exposures except for the third exposure for CS and *dumb*¹ flies (Figure 2.2 ).
Furthermore, I examined the sedative effect of ethanol on D1 dopamine receptor mutants with 70% ethanol. I exposed 4-5 days old \textit{dumb}^1 and \textit{dumb}^2 male flies along with the control CS for 6 consecutive days to 70% ethanol Flypub. MSTs for the first exposure were 34.10 ± 0.89 min, 36.48 ± 1.86 min and 32.92 ± 2.61 min for \textit{dumb}^1, \textit{dumb}^2 and CS, respectively (p > 0.05). Similar to 95% ethanol, all genotypes developed tolerance to the sedative effect with 70% ethanol. On the second exposure MST for \textit{dumb}^1 was 39.29 ± 1.74 min, \textit{dumb}^2 42.58 ± 1.30 min and CS 35.36 ± 1.67 min (not statistically different). Tolerance developed on the third and subsequent exposures to ethanol did not significantly change in \textit{dumb} flies while CS males showed decreased tolerance on the forth exposure (Figure 2.3).

Figure 2.2: Sedation profiles of \textit{CS}, \textit{dumb}^1 and \textit{dumb}^2 male flies exposed to 95% ethanol- \textit{CS} male flies (diamond, N=6), \textit{dumb}^1, (cross, N=6) and \textit{dumb}^2 (triangle, N=6) were exposed to 95% ethanol vapor in Flypub. There was no significant difference among the sedation of \textit{CS}, \textit{dumb}^1 and \textit{dumb}^2 flies on all exposures except for the third (P<0.05).
To investigate the role of DAT in the sedative effect of ethanol, I exposed \textit{fmn} mutants to both 95\% and 70\% ethanol along with \textit{CS} as a control. MST on the first exposure with 95\% ethanol in \textit{fmn} mutants was 18.98 ± 0.44 min, which was significantly lower than 22.90 ± 0.42 min in \textit{CS} (N=11, p <0.0001). On the second exposure \textit{fmn} flies were sedated with MST of 21.36 ± 0.30 min and \textit{CS} with 26.6 ± 0.67 min (N=11, p <0.0001). The trend was the same throughout the six days of ethanol exposure. Both \textit{fmn} and \textit{CS} developed tolerance which didn’t change significantly during the rest of exposures (Figure 2.4).
With 70% ethanol, \textit{fmn} and \textit{CS} male flies showed the similar trend (Figure 2.5).

The \textit{fmn} flies were sedated with MST of 23.32 ± 0.50 min and \textit{CS} flies with 29.59 ± 0.80 min (N=12, p < 0.0001). The second exposure with 70% ethanol yielded MST of 28.99 ± 0.59 min and 37.10 ± 0.54 min for \textit{fmn} and \textit{CS} flies, respectively (N=12, p < 0.0001). Tolerance was maintained relatively constant on the subsequent exposures (Figure 2.5).

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Figure 2.4: Sedation profiles of \textit{fmn} (N=11) and \textit{CS} (N=11) with 95% ethanol, General linear model revealed the significant effects of exposure, genotype and interaction (exposure effect \(F_{5,131} = 11.10\), p <0.0001, genotype effect \(F_{1,131}=282.68\), p <0.0001, significant difference by Tukey-Kramer tests in each exposure, interaction , \(F_{5,131}= 2.61\), p =0.028; n=11).
To examine the role of D2 receptors on the sedative effect of ethanol, I exposed D2 receptor mutant flies in the CS background to both 95% and 70% ethanol. Since the D2 mutant used in this study has a piggyBac carrying transgenic mini-white (mw) in the second intron of the dd2r gene on X chromosome, the CS flies carrying one copy of transgenic mw was used as a control along with CS. D2 receptor mutant flies showed MST of 28.1 ± 0.28 min on the first 95% ethanol exposure whereas CS showed MST of 22.5 ± 0.48 min and mw/+ 22.8 ± 0.62 min (Figure 2.6). I also exposed CS, mw/+ and D2 receptor mutant flies to 70% ethanol. MST of D2 mutant flies on the first exposure was 40.09 ± 0.58 min whereas MST of CS flies was 34.30 ± 1.49 min and mw/+ 33.40 ±

Figure 2.5: Sedation profiles of fmn & CS upon exposure to 70% ethanol. General linear model fmn (diamond, N=12) and CS (N=12) showed statistically significant difference in exposure and genotype effects (exposure effect, F_{5,143}= 29.58, p-value<0.0001, genotype effect, F_{1,143}=3888.71, p-value<0.0001, interaction, F_{5,143}=1.00, p-value= 0.422 ; N=12).
1.36 min (Figure 2.7). Thus with both 95% and 70% ethanol exposures, D2 mutant flies were more resistant to the sedative effect of ethanol. D2 mutant flies, nonetheless, developed tolerance similar to CS and mw/+ controls (Figure 2.6, Figure 2.7).

Figure 2.6: Sedation profiles of D2 mutant, CS and mw/+ in 95% Flypub. D2 receptor mutant males (triangle, N=12) showed MST higher than that of CS (diamond, N=6) or mw/+ (square, N=5). General linear model showed significant difference in MST of D2 compared to CS and mw/+, (genotype effect, $F_{3,137}=71.08$, $p<0.0001$, exposure effect, $F_{5,137}=9.74, p<0.0001$, interaction, $F_{15,137}=0.85$, $p=0.620$).
fmn and D2 receptor mutants showed the opposite phenotypes in the ethanol’s sedative effect. fmn mutants presumably have an increased dopamine level in synapses, resulting in enhanced dopamine neurotransmission. Thus, it seems that the enhanced sensitivity of fmn may be due to enhanced dopamine neurotransmission via D2 receptor since the flies defective in D2 showed decreased sensitivity. This needs to be verified by testing the double mutants defective both in fmn and D2 receptor. While D2 seems to be a major receptor mediating the sedative effect of ethanol, the fmn’s enhanced sensitivity was suppressed by D1 mutation since fmn;dumb1 double mutants showed MST comparable to that of CS or dumb1 (Figure 2.8). It is possible that D1 mutation

Figure 2.7: Sedation profiles of D2, CS and mw/+ in 70% Flypub. D2 mutant flies (triangle, N=12) showed significantly increased MST compared to CS (diamond, N=6) and mw/+ (square, N=5). General linear model revealed significant differences of genotype, exposure and interaction (genotype effect, $F_{22,143}=198.74$ p <0.0001; exposure effect $F_{5,143}=3.02$, p <0.0001; interaction $F_{10,143}=3.02$, p = 0.002).
compensates for excessive activation of D2 receptor in the \textit{fmn} background at least on the first ethanol exposure. However, this effect seems to diminish with additional ethanol exposure since MST of \textit{fmn;dumb} \textsuperscript{1} double mutants gradually approached to that of \textit{fmn} single mutant (Figure 2.8). In this experiment, MST of \textit{fmn} on the 6\textsuperscript{th} exposure was drastically reduced. Independent sets of experiments need to performed to address whether additional factors are involved in \textit{fmn}’s responses to ethanol.

We tested \textit{Elav-GAL4/UAS-dDA1;dumb} \textsuperscript{1} and \textit{ElavGAL4/+;dumb} \textsuperscript{2}/dumb \textsuperscript{1} (both lines express transgenic or induced dDA1 in all neurons in the \textit{dumb} mutant background, along with \textit{CS} and \textit{ElavGAL4/GAL80\textsuperscript{R};dumb} \textsuperscript{1} (carrying two copies of mw without transgenic dDA1 expression) as controls in 95% Flypub [Figure 2.9]. There were no significant differences between sedation profiles of all the 4 gentypes in all exposures.
2.4 Role of The Dopaminergic System in Ethanol-Induced Courtship Disinhibition

Upon chronic ethanol exposure of flies, the male flies start to show a distinct sexual behavior. Normally male flies actively court female flies and rarely court male flies. If a male attempts to court other male, the courted male strongly rejects the courting male. However under the influence of ethanol, male flies show frequent courtship toward other males and the courted males tend not to reject the courting male [Lee et al., 2008]. This ethanol-induced loss of courtship inhibition represents a type of cognitive
impulsivity and requires normal dopamine neurotransmission since the males flies with inhibited dopamine neuronal activity do not show this behavior [Lee et al.,2008].

Moreover, the extent of disinhibited intermale courtship increases with additional experience of ethanol, indicating that flies develop behavioral sensitization to the ethanol’s effect on courtship disinhibition[Lee et al.,2008]. In order to investigate the role of the dopaminergic system in disinhibited intermale courtship behavior, I examined dopamine receptor and transporter mutant flies in Flypub as described in Materials and Methods.

To investigate the role of the D1 dopamine receptor dDA1, I first exposed $dumb^1$ and $dumb^2$ flies with mutations in dDA1 to ethanol. Compared to wild type flies, $dumb^1$ flies showed significantly higher levels of intermale courtship in all ethanol exposures (1$^{\text{st}}$, 2$^{\text{nd}}$, 3$^{\text{rd}}$ and 6$^{\text{th}}$ exposures performed in 95% Flupub are shown in Figure 2.10) . Similar to $CS$ flies, the percentage of male flies engaged in disinhibited courtship progressively increased with additional ethanol exposure.
Similarly, when *dumb*¹, *dumb*², *CS* (control for *dumb*¹) and *mw* (control for *dumb*²) flies were exposed to 70% ethanol, both *dumb*¹ and *dumb*² flies showed significantly higher levels of intermale courtship compared to their controls in all exposures with normal sensitization (Figure 2.11). These observations suggest that dDA1 is important for courtship disinhibition but not for behavioral sensitization.

Figure 2.10: Courtship profile of *CS* (N=6) and *dumb*¹(N=6)-95% Etoh- *dumb*¹ males showed higher intermale courtship compared to *CS*. (ANOVA: exposure effect, F₃,₄₈=114.0, p<0.0001, genotype effect, F₁,₄₈=111.2, p<0.0001; interaction, F₃,₄₈=1.68; p<0.0001; N=6).
In order to investigate the role of D2 receptor in the ethanol-induced disinhibited intermale courtship, I exposed Cantonized D2 mutant flies to 95% ethanol along with CS and mw/+ males in the CS background as controls. The disinhibited courtship levels were not statistically significant between D2 mutant and the controls in individual exposures (Figure 2.12). This finding indicates that D2 receptors may not be involved in intermale courtship behavior induced by ethanol. It is noteworthy to mention that D2 mutants in the w background showed impaired behavioral sensitization with normal induction of courtship disinhibition under chronic ethanol exposure (experiments performed by other
students in Dr. Han’s lab). It is possible that D2 mutant phenotype may be sensitive to the genetic background and the w background sensitizes the effect of D2 mutation.

To understand the role of dopamine transporter in inter male courtship, I examined *fumin* (DAT mutant) flies in 95% or 70% ethanol-containing Flypub. Surprisingly, *fumin* flies showed significantly lower levels of inter-male courtship compared to the control CS flies in both ethanol concentrations (Figure 2.13, Figure 2.14).

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Figure 2.12: Effects of D2 receptor on the disinhibited male courtship behavior of flies under the influence of 95% ethanol. Performing two-way ANOVA didn’t reveal any significant difference between the level of courtship in D2 mutant flies (N=12) comparing to the wild type CS (N=6) and *mw/+* (N=6) as a control.

To understand the role of dopamine transporter in inter male courtship, I examined *fumin* (DAT mutant) flies in 95% or 70% ethanol-containing Flypub. Surprisingly, *fumin* flies showed significantly lower levels of inter-male courtship compared to the control CS flies in both ethanol concentrations (Figure 2.13, Figure 2.14).
Figure 2.13: Intermale courtship of \textit{fnn} and \textit{CS} in 95\% Etoh Flypub. Statistical analysis revealed the significant difference between \textit{CS} (N=11) and \textit{fnn} (N=11) on both genotype and exposure except for the first exposure, in which no courtship activity was observed (General linear model; exposure effect, $F_{3,87}=169.90$, $p<0.0001$; genotype effect, $F_{1,87}=310.71$, $p<0.0001$; interaction, $F_{3,87}=68.72$, $p<0.0001$).
To investigate whether the fmn’s phenotype is rescued by dDA1 receptor mutation and vice versa, I tested the fmn; dumb\(^1\) double mutant males along with fmn, dumb\(^1\) and CS males in 95% Flypub. As shown previously, the levels of intermale courtship of dumb\(^1\) were significantly higher than those of CS in all exposures; however, the levels of male-male courtship of fmn were significantly lower than CS. Notably, the levels of disinhibited courtship of fmn;dumb\(^1\) and CS were comparable (Figure 2.15). This suggests that the abnormal ethanol-induced inter male courtship in fmn mutants may be due to overly activated D1 receptor. Likewise, the elevated inter male courtship in D1

Figure 2.14: Intermale courtship of fmn and CS in 70% Etoh Flypub. We exposed fmn (N=12) and CS (N=12) to 70% ethanol and we observed significant difference between genotype and in each exposure. (General Linear Model, genotype effect \(F_{1,95}=174.18, P<0.0001\), exposure effect, \(F_{3,95}=86.24, P<0.0001\); interaction \(F_{3,95}=37.58, P<0.0001\)).
mutants dumb may be overcome by enhanced dopamine neurotransmission via other dopamine receptor type(s) such as D5 receptor DAMB or D2 in *fmn* mutants.

As shown above, the mutant male flies defective in the D1 receptor dDA1 showed the elevated ethanol-induced inter male courtship. To confirm that this phenotype is indeed mediated by loss of dDA1 function, I conducted rescue experiment. To restore dDA1 expression in the CNS, I used the pan-neuronal Elav-GAL4 driver and UAS-dDA1 or *dumb* carrying the piggyBac with UAS that is inserted in the first intron of the dDA1

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2.15: Courtship profile of double mutant *fmn; dumb* along with *dumb*, *fmn* and CS as controls- 95% EtOH. I exposed double mutant *fmn; dumb* (N=12) along with *dumb* (N=8), *fmn* (N=6) and CS (N=12) to 95% ethanol in Flypub. Statistical analysis of intermale courtship percentage under the influence of 95% ethanol showed significant effects of exposure and genotype between the double mutant *fmn; dumb* and *fmn* and *dumb*. (General linear model, genotype effect, F \(_{3,151}=129.80\), p<0.0001, exposure effect, F \(_{3,151}=205.81\), p <0.0001 and interaction, F \(_{9,151}=10.38\), p <0.0001).

As shown above, the mutant male flies defective in the D1 receptor dDA1 showed the elevated ethanol-induced inter male courtship. To confirm that this phenotype is indeed mediated by loss of dDA1 function, I conducted rescue experiment. To restore dDA1 expression in the CNS, I used the pan-neuronal Elav-GAL4 driver and UAS-dDA1 or *dumb* carrying the piggyBac with UAS that is inserted in the first intron of the dDA1
gene [Kim YC at al, 2006]. The UAS in \textit{dumb}^2 upon binding to GAL4 activates the endogenous dDA1 [Kim YC at al, 2006]. \textit{Elav-GAL4/UAS-dDA1;dumb}^1 and \textit{Elav-GAL4/+;dumb}^2/dumb^1 along with \textit{ElavGAL4/GAL80ts;dumb}^1 (a control with matching \textit{mw} copy number and with no dDA1 expression) and \textit{CS} as controls were subjected to 95\% ethanol exposure. Similar to \textit{dumb} mutants, the inter male courtship levels of \textit{ElavGAL4/GAL80ts;dumb}^1 male flies were significantly higher than those of \textit{CS} [Figure 2.16]. Inter male courtship levels of \textit{Elav-GAL4/+;dumb}^2/dumb^1, however, were comparable to those of \textit{CS}, indicating that the restored dDA1 expression in the dumb mutant brain rescued the dumb’s phenotype. On the other hand, the courtship levels of \textit{ElavGAL4/UAS-dDA1;dumb}^1 were similar to those of \textit{ElavGAL4/GAL80ts;dumb}^1 rather than \textit{CS}. It is possible that the level of transgenic dDA1 expressed via the UAS-dDA1 transgene may not be sufficient to rescue the dumb’s phenotype. Future studies using two copies of UAS-dDA1 may help clarify it. Taken together, the elevated disinhibited courtship in dumb mutants was rescued by reinstating endogenous dDA1 expression in the CNS via UAS in \textit{dumb}^2 and dDA1 may be crucial for suppressing ethanol-induced courtship disinhibition.
3.4 Conclusion

Ethanol has several effects on sexual behavior of human and animals. In humans, it increases the sexual arousal and motivation and reduces the sexual performance [Markos et al., 2005]. Furthermore, chronic ethanol consumption could be associated with disinhibited sexual behavior. A study conducted on rats showed that ethanol can reestablish the sexual behavior of male rats that had been trained to suppress
their sexual behavior towards unresponsive females. On the contrary, Scott et al in 1994, failed to observe the disinhibited sexual behavior with ethanol treatment [Scott MP et al, 1992]. It has been previously documented that male courtship behavior and sexual orientation are established during development by the gene FruM. However, the disinhibited courtship discussed here is a post development effect of chronic ethanol on sexual behavior of *Drosophila melanogaster*.

The disinhibited intermale courtship is induced upon chronic ethanol exposure in wild type *Drosophila*. Dopamine is suggested as a key molecule mediating this distinct sexual behavior [Lee et al.,2008].

In order to unveil the mechanisms that dopamine mediates the ethanol-induced behaviors, I investigated different dopamine receptor mutant flies. Dopamine receptors consist of five subtypes in mammals (D1-5), from which only D1, D2 and D5 are present in *Drosophila*. D1 receptor mutant flies showed higher levels of ethanol-induced intermale courtship compared to wild type flies while they showed normal sensitivity to the sedative effect of ethanol. Thus D1 receptor plays a selective role in disinhibited sexual behavior but not in sedation induced by ethanol.

D2 receptor mutant flies showed reduced sensitivity to both 95% and 70% ethanol. This is consistent with previous observations that reduced sensitivity of D2 receptor is associated with higher susceptibility to alcoholism [Kraschewski et al, 2009]. D2 receptor mutant flies, on the other hand, showed comparable levels of the ethanol-induced intermale courtship compared to the wild type flies, indicating that D2 receptor is not involved in this phenomenon. Lack of involvement of D2 receptor in male-male courtship was also shown by Liu’s group [Liu et al., 2008].
The enhanced level of dopamine has been reported to increase the male-male courtship in the absence of ethanol in *Drosophila* [Liu et al., 2008]. DAT mutant, *fumin* flies, which likely have enhanced dopamine neurotransmission, did not show inter male courtship in the absence of ethanol. Under the influence of ethanol, the level of disinhibited courtship was much lower in *fumin* flies compared to the wild type. The *fumin* flies exhibit higher locomotor activity compared to the CS, which might be responsible for reduced inter male courtship.
Chapter 3

Role of amnesiac in Ethanol-Induced Courtship Disinhibition

3.1 Introduction

It has been documented that cAMP pathway has an important role in acute and chronic ethanol exposure. Although acute exposure to ethanol has been shown to increase the amount of cAMP, chronic exposure has the opposite effect and decreases the level of cAMP. In acute ethanol exposure, the enhanced level of cAMP would lead to activation of protein kinase A (PKA) which in turn phosphorylates target proteins such as cAMP response element binding protein (CREB). During chronic ethanol exposure, the decrement in cAMP level appears to be the result of desensitization of G stimulating coupled proteins or elevation of G inhibitory protein level [Bellen et al., 1998].

It has been previously shown that binding of amnesiac to its receptor would increase the level of cAMP in the cytoplasm. Increasing the level cAMP could be induced by activation of adenylate cyclase (AC). AC is encoded by rutabaga gene that was also found to be related to learning and memory. DCO is also another mutant that encodes cAMP dependent protein kinase C (PKA-C). Furthermore, the higher ethanol sensitivity of *rutabaga* (AC) and DCO is similar to the ethanol sensitivity of *amnesiac* flies [Davis RL et al, 1996].

On the other hand, *dunce* mutant flies that lack phosphodesterase enzyme (PDE) and subsequently have increased level of cAMP, showed ethanol sensitivity similar to
wild type flies [Byers D. et al, 1981]. The in vitro observation on individual cells has also been shown that acute ethanol exposure would increase the level of cAMP. Thus, inability to upregulate the level of cAMP has been proposed to be the basis of ethanol sensitivity [Moore et al., 1998].

To further investigate the effect of cAMP level on ethanol sensitivity, Moore et al fed the amnesiac mutants with forskolin, which activates the adenylate cyclase. The activation in adenylate cyclase activity restores the ethanol sensitivity of amn and rut mutants. Moreover, inhibition of protein kinase A (PKA) in wild type flies has been accompanied by an increase in ethanol sensitivity. Taken all together, cAMP signaling pathway plays a key role in regulation of ethanol sensitivity in flies [Moore et al., 1998]. Studies done in humans and mammals have also demonstrated the central role of cAMP pathway in regulating ethanol sensitivity. The level of AC was significantly decreased in platelets and lymphocytes of alcoholics even after long deprivation from alcohol [Tabakoff et al, 1988].

Moore et al in 1998 isolated various mutants that were sensitive to ethanol. They found cheapdate (chpd) mutant flies that are allelic to amnesiac gene. The cheapdate mutant is a P-element insertion mutant that has been isolated based on its increased sensitivity towards intoxication effects of ethanol. The insertion of chpd gene is located on the X chromosome (19A1-2), the same location which amnesiac gene has been mapped previously. Furthermore, the P-element in chpd mutants was inserted within the open reading frame of Amn gene C-terminal to the PACAP and GHRH homologous regions [Moore et al., 1998]. DNA sequence of Amn locus showed an ORF of 541 bp beginning with the AUG at position 2231 and terminating at position 2771. The amino
acid product of Amn gene has a 180 amino acid based on conceptual translation. According to genetic and molecular data, chpd is considered another allele of Amn and they named it amn<sup>chpd</sup> [Moore et al., 1998].

Additional amn<sup>l</sup> alleles consist of amn<sup>28A</sup> and amn<sup>X8</sup> which also show ethanol sensitivity similar to amn<sup>l</sup>. Ethanol sensitivity phenotype of P-element induced allele amn<sup>28A</sup> was undistinguishable from amn<sup>chpd</sup>. Amn<sup>X8</sup> mutant that has a complete deletion amn locus showed even more sensitivity towards sedative effects of ethanol compared to other amn alleles [Moore et al., 1998].

3.2 Material and Methods

3.2.1 Fly Stock

We used Canton S, from Bloomington center as a wild type strain. amn<sup>l</sup>, amn<sup>28A</sup>, amn<sup>chpd</sup> were kindly provided by Dr. Heberlien (University of California San Francisco). We obtained the UAS-amn and c316 flies from Dr. Saito (Tokyo Metropolitan Institute for Neuroscience).

3.2.2 Fly Culture

We used Standard cornmeal to rear the flies at 25° C. The flies were kept at 12 h light/12 h dark photoperiod with 50% relative humidity. Male flies were used for the behavioral assay. Flies within one to two days after eclosion were collected under CO2 anesthesia and transferred to food vials. We used 33 males as a group and aged them for
4-5 days after eclosion for the behavioral assay. Each set of ethanol exposure contains 6 groups of 33 males (N=6). In order to use control for the observed behavior, we divided each set to 3 groups of experimental males and 3 groups of control male flies.

3.2.3 Behavioral Assay

Behavioral assay was performed as previously discussed in chapter 2. Statistical analysis was done using Minitab 15 (Minitab Inc., State College, PA, USA). All data are reported as mean ± Standard error of the mean. We used two-tailed student t-test to compare the means of two groups. In case there were more than two groups to compare, we performed analysis of variance (ANOVA).

3.3 Results

3.3.1 Tolerance Development After Chronic Ethanol Exposure

The mean sedation time (MST) of $amn^l$ male flies on the first 95% ethanol exposure, was 18.54 ± 021 min compared to 20.6 ± 0.32 min for Canton-S (CS). Both $amn^l$ and CS flies developed tolerance on the second ethanol exposure, 22.09 ± 0.19 min and 24.19 ± 0.30 min for $amn^l$ and CS, respectively. The tolerance developed after exposing them to ethanol didn’t change significantly during consecutive ethanol exposures (Figure 3.1).
Furthermore, we observed the behavior of amn flies when exposed to 70% ethanol for 6 consecutive days. On the first day, amn flies had the MST of 26.04 ± 0.49 min versus CS which had the MST of 28.88 ± 0.35 min. They both developed tolerance over the next exposures, which didn’t change significantly over the next exposures (Figure 3.2).

Figure 3.1: Sedation profile of CS and amn after exposure to 95% Etoh- amn (dash, N=12) and CS (diamond, N=12) showed significant difference between exposures and genotype. (General linear model statistics, genotype effect, F₁,₁₃₇=186.52, p<0.0001, exposure effect, F₅,₁₃₇=4.81, p<0.0001, interaction, F₅,₁₃₇=0.70, p=0.623).
In order to establish the role of Amn gene in ethanol sensitivity, we tested other amn alleles. The \textit{amn\textsuperscript{chpd}} flies showed the same sedation profile as \textit{amn\textsuperscript{l}}. On the first exposure, \textit{amn\textsuperscript{chpd}} had the MST of 17.13 ± 0.18 min where CS sedated with the mean sedation time of 22.52 ± 0.18 min. On the second exposure both \textit{amn\textsuperscript{chpd}} and CS, developed tolerance to sedative effects of ethanol which didn’t change significantly during the following exposures (Figure 3.3 ). The same as exposing flies to 95% ethanol, a similar trend of results was also seen with 70% ethanol.

Figure 3.2: Sedation profile of \textit{amn\textsuperscript{l}} and CS after exposing to 70% Etoh- statistic analysis by general linear model revealed significant difference between \textit{amn} (dash, N=11) and CS (diamond, N=11) mean sedation time based on exposure, genotype and their interaction( exposure effect, F\textsubscript{5,137}=3.92, p<0.0001, genotype effect, F\textsubscript{1,137}=159.69, p< 0.0001, interaction, F\textsubscript{5,137}=3.95, p=0.002).
Figure 3.3: Sedation profile of $amn^{chpd}$ and CS-95%. The $amn^{chpd}$ (N=11) and CS (N=11) had significant difference in their sedation profile (general linear model statistic, exposure effect, $F_{5,131}=6.70$, $p<0.0001$, genotype effect, $F_{1,131}=834.65$, $p<0.0001$, interaction, $F_{5,131}=3.333$, $p=0.008$).
However, *amn*<sup>28A</sup> showed completely different sedation profile than *amn*<sup>chpd</sup> and *amn*<sup>I</sup>. The mean sedation time of *amn*<sup>28A</sup> on the first exposure to 95% ethanol was 23.45±1.39 min, which was significantly higher than the MST of *CS* on the first exposure, 21.44±1.44 min (P-Value = 0.003) (Figure 3.5). The ethanol sensitivity of *amn*<sup>28A</sup> was previously addressed by using different device called inebriometer and measuring the elution time of the flies [Moore et al., 1998]. However, we exposed the flies in a novel device Flypub, which has different condition and parameter than inebriometer and also we used different parameter (MST) to assess the ethanol sensitivity of the *amn*<sup>28A</sup> flies. Furthermore, the exact genotype of *amn*<sup>28A</sup> which we used should be determined in order to be able to state the conclusion. The later argument was based on

![Sedation profile of CS and amn<sup>chpd</sup> after exposure to 70% Etoh. The amn<sup>chpd</sup> (square, N=11) and CS (diamond, N=11) had significant difference in their sedation profile (general linear model statistic, exposure effect, F<sub>5,131</sub>=10.90, p<0.0001, genotype effect, F<sub>1,131</sub>=1532.86, p<0.0001, interaction, F<sub>5,131</sub>=0.496, p=0.496).](image-url)
the fact that the flies which we got were examined almost 10 years after the first experiments done by Moore et al.; during these 10 years, there might be some mutation happening in the gene which could have affected the results we observed here.

3.4 Rescuing ethanol sensitivity defect of \textit{amn}^1

The data presented in previous section suggested that Amn gene disruption might be responsible for ethanol sensetivity of \textit{amn} mutant male \textit{Drosophila}. In order to evaluate this hypothesis conclusively, we tried to rescue \textit{amn}^1 mutants with UAS/GAL4

Figure 3.5: Sedation profile of \textit{amn}^{28A} (N=11) and CS (N=11) under the influence of 95% ethanol. Performing a general linear model statistic analysis showed that there is significant difference between mean sedation time of \textit{amn}^{28A} and CS flies base on different exposure and genotype (exposure effect, F_{5,131}=41.20, p<0.0001, genotype effect, F_{1,131}=22.45, p<0.0001, interaction, F_{5,131}=0.58, p=0.713).
system. We exposed \textit{amn^1;UASamn/amnGAL4}, \textit{amn^1;UASamn/+}, \textit{amn^1;amnGAL4/+}, \textit{amn^1;UASamn}, \textit{amn^1;amnGAL4}, \textit{amn^1} and CS male flies to 95\% ethanol in order to see
if the ethanol-sensitive phenotype of amn1 flies could be rescued (Figure 3.6). We

![Figure 3.6: Sedation profile of amn1;UASamn/amnGAL4, amn1;UASamn/+ , amn1;amnGAL4/+, amn1;UASamn/UASamn, amn1;amnGAL4/amnGAL4, amn1 and CS male flies after exposure to 95% ethanol. General linear model revealed the significant effects of exposure and genotype (genotype effect, F_{6,287}=35.72, p<0.0001, exposure effect, F_{5,287}=8.18, interaction, F_{30,287}=0.83, p=0.723).]

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MST ± SEM (min)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>amn1;UASamn/amnGAL4</td>
<td>23.21 ± 0.58</td>
<td>16</td>
</tr>
<tr>
<td>amn1;UASamn/+</td>
<td>22.87 ± 1.02</td>
<td>6</td>
</tr>
<tr>
<td>amn1;amnGAL4/+</td>
<td>23.38 ± 0.88</td>
<td>3</td>
</tr>
<tr>
<td>amn1;UASamn</td>
<td>22.14 ± 0.58</td>
<td>3</td>
</tr>
<tr>
<td>amn1;amnGAL4/+</td>
<td>22.53 ± 0.58</td>
<td>8</td>
</tr>
<tr>
<td>amn1;UASamn/+</td>
<td>21.70 ± 0.41</td>
<td>7</td>
</tr>
<tr>
<td>amn1</td>
<td>18.93 ± 0.51</td>
<td>5</td>
</tr>
</tbody>
</table>
observed that MST of amn1;UASamn/amnGAL4, amn1;UASamn/+, amn1;amnGAL4/+, amn1;UASamn/UASamn, amn1;amnGAL4/amnGAL4, and CS was significantly higher than amn1 on all the exposures. However, there was no significant difference between amn1;UASamn/amnGAL4, amn1;UASamn/+, amn1;amnGAL4/+, amn1;UASamn/UASamn, amn1;amnGAL4/amnGAL4, and CS.

3.4.1 Role of amnesiac Gene in Male-Male Courtship

Recorded videos of exposed flies to ethanol were carefully reviewed in order to calculate the courtship percentage of male-male courtship of the flies. The maximum number of flies were scored in each 30 seconds and the average of consecutive periods were used to present the percentage of the disinhibited courtship. In order to find the role of amnesiac gene in disinhibited courtship we exposed amn1 mutants along with CS as the control to ethanol. The intermale courtship percentage of amn1 flies was significantly higher than the percentage courtship in CS flies both with 95% and 70% ethanol (Figure 3.7, Figure 3.8).
Figure 3.7: courtship of amn\textsuperscript{i} and CS-95\% Etoh. The amn\textsuperscript{i} mutant male flies (N=12) showed higher level of courtship compared to the control, CS (N=12) (general linear model, exposure effect, F\textsubscript{1,95}=100.21, p<0.0001; genotype effect, F\textsubscript{3,95}=199.11, p<0.0001; interaction, F\textsubscript{3,95}=3.59, p=0.017).
In order to establish the role of amnesiac gene in the disinhibited courtship, we also exposed other amnesiac alleles, amn$^{28A}$ and amn$^{chpd}$ to ethanol. The percentage of flies engaged in male-male courtship was also significantly higher in amn$^{28A}$ compared to CS (Figure 3.9).

Figure 3.8: Courtship amn$^1$ and CS-70% Etoh. amn$^1$ mutant male flies (N=12) showed higher level of courtship compared to CS (N=12) (General linear model, exposure effect, F$_{3,95}=391.23$, p <0.0001, genotype effect, F$_{1,95}=461.88$, p <0.0001, interaction, F$_{3,95}=22.91$, p <0.001).
However, the courtship level between males of $amn^{28A}$ was lower than of $CS$ flies. Under exposure of 95% ethanol the percentage of courtship in male flies in all the evaluated exposures were significantly lower than the control (Figure 3.10). Furthermore, we observed the same behavior under the influence of 70% ethanol. The $amn^{chpd}$ flies showed lower level of courtship in all consecutive ethanol exposures compared to $CS$ (Figure 3.11).

Figure 3.9: Courtship profile of $amn^{28A}$ and $CS$ -95% Etoh. Statistical analysis with ANOVA showed that there is significant difference between the percentage courtship of $amn^{28A}$ (N=6) and $CS$ (N=6) (general linear model, exposure effect, F$_{1,87}$=110.37, p <0.001, genotype effect, F$_{3,87}$=177.65, p <0.0001, interaction, F$_{3,87}$=13.32, p <0.0001).
Figure 3.10: Courtship profile of CS and amn

exposed amn

mutant (N=12) and CS male flies (N=12) to 95% ethanol and compared the percentage of inter male courtship. The amn

male flies showed significantly higher level of courtship compared to the wild type flies (exposure effect, F_{3,87}=291.89, p<0.0001, genotype effect, F_{1,87}=515.33, p<0.0001, interaction, F_{3,87}=158.23, p<0.0001).
To further evaluate the role of Amn gene in disinhibited courtship, I tried to rescue amn mutants by using the UAS/GAL4 system. I exposed amn\textsuperscript{l};UASamn/amnGAL4, amn\textsuperscript{l};UASamn/+, amn\textsuperscript{l};amnGAL4/+, amn\textsuperscript{l};UASamn/UASamn, amn\textsuperscript{l};amnGAL4/amnGAL4, amn\textsuperscript{l} and CS male flies to 95% ethanol and measured the percentage of male flies engaged in courtship (Figure 3.12). I observed that the courtship percentage of amn\textsuperscript{l} male flies were significantly higher than amn\textsuperscript{l};UASamn/amnGAL4, amn\textsuperscript{l};UASamn/+, amn\textsuperscript{l};amnGAL4/+,

Figure 3.11: Courtship profile of CS and amn\textsuperscript{chpd} in 70% Etoh Flypub. Surprisingly, amn\textsuperscript{chpd} (N=12) showed lower level of intermale courtship compared to the wild type CS (N=12) (general linear model, genotype effect, $F_{1,87}=543.99$, $p<0.0001$, exposure effect, $F_{3,87}=226.83$, $p<0.0001$, interaction, $F_{3,87}=177.92$, $p<0.0001$).
significant differences between \( \text{amn}^1;\text{UASamn}/\text{amnGAL4} \), \( \text{amn}^1;\text{UASamn}/+ \), \( \text{amn}^1;\text{amnGAL4}/+ \), \( \text{amn}^1;\text{UASamn}/\text{UASamn} \), \( \text{amn}^1;\text{amnGAL4}/\text{amnGAL4} \) and CS.

3.5 Conclusion

Multiple studies have showed that proper cAMP signaling is required for normal alcohol sensitivity. The cAMP signaling pathway mainly consists of three components. First, a transmembrane receptor, which binds to external signal; second, a G-protein; third, a G-protein stimulated Adynylate cysclave, which converts ATP to cAMP. Several experiments proposed that Amn gene would enhance the cAMP level based on its

Figure 3.12: Inter male courtship in \( \text{amn}^1;\text{UASamn}/\text{amnGAL4} \), \( \text{amn}^1;\text{UASamn}/+ \), \( \text{amn}^1;\text{amnGAL4}/+ \), \( \text{amn}^1;\text{UASamn}/\text{UASamn} \), \( \text{amn}^1;\text{amnGAL4}/\text{amnGAL4} \), \( \text{amn}^1 \) and CS male, after exposure to 95% ethanol in flypub- General linear model revealed the significant effects of exposure, genotype and interaction (exposure effect, \( F_{3,191}=391.75 \), \( p<0.0001 \); genotype effect, \( F_{6,191}=36.39 \), \( p<0.0001 \); interaction, \( F_{18,191}=47 \), \( p=0.001 \)).
homology to neuropeptides found in mammals (like Growth Hormone and PACAP) that couples with adenylate cyclase. Furthermore, suppressing the dnc female flies sterility is considered another reason that \textit{amn} mutant would increase the cAMP level [Moore et al., 1998]. Although it was previously shown that \textit{amn} mutant have increased sensitivity to ethanol but its role in the disinhibited courtship after recurrent daily ethanol exposure still needs more clarifications.

We observed that \textit{amn} mutants have some diverse reaction to ethanol that makes it rather difficult to interpret. Both \textit{amn}^{1} and \textit{amn}^{chpd} showed increased sensitivity to sedative effects of ethanol but \textit{amn}^{28A} on the contrary showed higher resistance to sedative effects of chronic alcohol exposure. However, Moore et al. found different results in an inebriometer; they reported that \textit{amn}^{28A} phenotype is similar to other \textit{amn} mutants in regards to ethanol sensitivity. There might be several explanations for this phenotype of \textit{amn}^{28A} flies. We exposed the flies in a novel device Flypub, which has different condition and parameter than inebriometer which were used by Heberlein’s group [Moore et al., 1998].

The \textit{amn}^{1} mutants showed higher level of disinhibited intermale courtship. The results we reported were consistent in \textit{amn}^{28A} which has been shown to have significantly higher level of male-male courtship. However, there was in consistency in the courtship level seen in \textit{amn}^{chpd} flies. The \textit{amn}^{chpd} males showed significantly lower level of disinhibited courtship upon chronic ethanol exposure. Although, \textit{amn}^{chpd} flies have similar response to ethanol compared to wild type flies, these mutants have higher locomotor activity upon exposure to ethanol and they reach maximal hyperactivity more
quickly than the CS [Wolf et al., 2002]. We suggest that the higher locomotor activity of

$amn^{chpd}$ flies might be the responsible for the reduced inter male courtship.
4.1 Summary of Results and Conclusion

Ethanol is a drug of abuse that has been around for centuries, nonetheless the detailed effects on the behavior and cellular mechanisms underlying these effects still needs to be clarified. Chronic ethanol administration has diverse effects on different aspects of behavior, from sedation and tolerance development to altered sexual behavior. Recurring exposure to ethanol would cause the male *Drosophila* to court each other. Male flies that haven’t been exposed to ethanol would vigorously reject other males and tend to strongly court females. On the other hand, the male flies that have been exposed to toxic amount ethanol upon daily exposure had shown inter male courtship. This increased courtship is confined to male flies, since females didn’t show enhanced courtship level after chronic ethanol exposure [Lee et al., 2008].

Impaired level of dopamine in flies has been reported to cause enhanced level of disinhibited courtship [Lee et al., 2008]. Taken together, the study presented here confirmed the critical role of dopamine and further identified the D1 dopamine receptor dDA1 as a major receptor type mediating the ethanol-induced disinhibited inter male courtship. Ethanol enhances the level of dopamine in different brain areas in rodents and human beings [Tabakoff et al., 1988]. Ethanol can also make adaptive changes in dopamine receptor and dopamine transporter [Diana et al., 1996]. Thus, this study is
consistent with the findings in rodents and humans, and suggests abnormal dopamine activity may be responsible for ethanol-induced behavioral disinhibition in humans.

The Amn gene which is believed to code PACAP (Growth hormone like protein) has been previously documented to play an important role in acute and chronic ethanol exposure [Bellen et al., 1998]. The study presented here, shows the important role of cAMP level and amn gene in disinhibited courtship in male flies under the influence of ethanol.

4.2 Future Work

In order to completely understand the cellular mechanism underlying ethanol induced disinhibited courtship, more experiments would be necessary such as experiments investigating the role of other neurotransmitters. Serotonin could be the next neurotransmitter to evaluated followed by role of Gluamic acid and other neurotransmitters such as GABA.

In this thesis we showed that dopamine level is important in regulating the the sexual behavior and exibiting the disinhibited courtship after chronic ethanol exposure. However, more investigations are necessary to establish which part of the brain these specific neurotransmitters would cause their effects. There might be some other genes also involved in this behavior that should be identified next.

In my opinion, it would be also interesting to evaluate the sexual performance of the male flies under the influence of ethanol. It has been reported that ethanol would
decrease sexual performance and increase sexual desire. It should be really interesting to evaluate the sexual performance after chronic ethanol exposure of the male flies.

Moreover, the same experiments on rats and mice could give us some complement results that would help to better evaluate the role of mamalian genes in disinhibited courtship and the effects of ethanol. It will also give a better insight on the sexual behavior and performance that will enable the scientists to come to a better therapy for the disorders in sexual behavior, drug addiction and also its chronic effects on human health.
Bibliography


Bellen HJ. The fruit fly: a model organism to study the genetics of alcohol abuse and addiction. Cell. 1998 Jun 12; 93(6):909-12


Heberlein U., Genetics of Alcohol-Induced Behaviors in *Drosophila*, Alcohol Research & Health, 2000 (24)185-188.


