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## A modified method to assay the effects of ethanol on the behavior of *Drosophila melanogaster*.

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### Abstract

For over a long period of time, many scientists have frequently studied the fruit fly *Drosophila melanogaster* as a model organism to help elucidate the complex mechanisms that govern development and behavior.  Recent advances in scientific research have enabled us to discover that certain strains of *Drosophila* are not only tolerant towards concentrations of alcohol, but also display many ethanol-induced behaviors resembling intoxication (*e.g.*, loss of motor control).  Hence, *Drosophila* makes an ideal model to study the effects of alcohol and deduce the neural circuitry involved in producing its intoxicating and rewarding effect. In the present study varying concentrations of ethanol were administered and its effect on the larval locomotion (Larval crawling assay), adult climbing ability (RING assay), and courtship behavior (Courtship and Mating assay) were assessed.  It was found that after short term exposure of ethanol, larvae were found to have a decreased locomotor activity at 10% ethanol concentration; adult flies had shown a biphasic reaction towards the effect of ethanol.  Mating behavior was affected by ethanol, with a reduction in the Courtship Index for flies that had been exposed to 20% ethanol (loss of postural control and instability in balance was observed).  Most of the circuits governing these behaviors involve the inhibition and excitation of certain neurotransmitters, which are conserved between humans and flies.  These results indicate that studies using *Drosophila* as a model system may help in understanding how ethanol influences behavior, which is vital to decipher the mechanisms of action of ethanol and alcoholism.  Keywords: *Drosophila melanogaster*; alcohol; behavior; larva.

### Introduction

The human society has resorted to the use of alcohol for a variety of reasons.  For more than a thousand years, documentation of its use as a part of food production, medicine, mood changers, and also as an intoxicant has been kept.  Along with their uses, the adverse effects of alcohol have also been documented to as far as written records have existed.  However the mechanisms responsible for alcohol related behavior and alcohol addiction are still poorly understood.

Complex genetic and environmental factors contribute to a predisposition to drug addiction.  The ability to modulate the genetic conditions, which are likely multigenic and heterogeneous make the process of identifying specific genes responsible for addiction a difficult task.

As a model organism *Drosophila* has been instrumental in providing insights into the various molecular (Ulrike, 2000) and neural mechanisms underlying addiction and intoxication (Devineni, 2000).

Along with *Drosophila,* other invertebrate organisms such as *C. elegans* have also been used to understand the mechanisms of action of various drugs (Boris Tabakoff, 2000).

*1.1 Mesolimbic-dopamine pathway*

Various neural circuits are responsible for the "rewarding effect" produced due to alcohol consumption.  Among the many neural circuits that are excited (or) suppressed, the mesolimbic dopamine pathway is most widely studied in association with drug addiction in humans (Piercea, 2000).  The limbic nuclei which primarily consists of the amygdala, hippocampus, and medial prefrontal cortex sends glutamatergic projections into the nucleus accumbens.  The nucleus accumbens is composed of the limbic sub region (or the shell) and the motor sub region (or the core).  These have two major outputs that send GABAergic projections into the ventral pallidum and the ventral tegmental area (substantia nigra).  The GABAergic efferents are further sent to the medial dorsal thalamus.  This stimulates the medial dorsal thalamus to provide glutamatergic projections to the medial prefrontal cortex, thus closing the limbic circuit.

The glutamatergic receptor, also known as the most abundant excitatory receptor in the brain, is present mostly in the hippocampal regions, which is responsible for the formation and storing of memory.  Dopamine acts through the limbic component of the basal ganglia.  Dopaminergic neurons in the VTA innervate most of the components of the limbic circuit, namely: the nucleus accumbens, amygdala, hippocampus, mPFC, and ventral pallidum.  Thus any changes in the transmission of dopamine plays a pivotal role in modulating the information flow through the various parts of the limbic circuit (Steven, 2005).

As discussed earlier, in mammals dopamine is an important regulator of the mesolimbic dopamine pathway.  Many of these dopaminergic cells have been shown to illicit much ethanol-related behavior by acting on various brain regions.  In *Drosophila,* the function of dopamine in regulating ethanol induced behavior is localized to a pair of dopaminergic neurons (DopR) projecting or expressing neurons, in the ellipsoid body of the central complex.  This region is responsible for visual and locomotor behavior, arousal, and memory in the fly.  The ellipsoid body also regulates sedation, sensitivity, and tolerance caused due to ethanol.  While most of the neurons innervate the ellipsoid body, the others terminate in the mushroom body, a brain structure responsible in olfactory learning and processing.  Neurotransmission of the mushroom body neurons is required for the behaviors seen due to ethanol-induced hyperactivity and conditioned ethanol preference.  Both of these behaviors are mediated in specific sub regions within the mushroom body by neurons (Kaun, 2012).

The neuranatomical organization of the fly and mammalian brain is quite different.  Hence it is not only difficult but also a tedious task to draw parallels between the neural regions and circuits involved in the ethanol induced behaviors in flies and mammals.

In mammals various brain centers such as the VTA, amygdala, ventral pallidum are involved in the mesolimbic pathway;  however, it is unclear if structures equivalent to these are found in the fly brain.

As seen earlier the brain structures that are involved in various ethanol induced responses are the ellipsoid body and the mushroom body.  The NPY/NPF system, a neuropeptidogeric system, is also found to regulate ethanol responses flies and rodents (Boris Tabakoff, 2000).

To understand further the effect of ethanol on the behavior of *Drosophila melanogaster,* a top down experimental approach was taken.  In this study we used various neuro-behavioral assays adapted from (Nichols, 2012).  The assays conducted were:

1. Larval crawling assay
2. Rapid Iterative Negative Geotaxis Assay
3. Courtship and Mating Assay.

Here we extended previous approaches utilized to understand the effect of ethanol in *Drosophila melanogaster*.

**Materials and Methods**

*Drosophila melanogaster* (wild type) were cultured in standard growth media with a 12 hr light and dark cycle.  Flies were transferred into fresh autoclaved vials once in every 3 days.  Each vial contained

approximately 35 flies.  The neurobehavioral assays performed were adapted from (Nichols, 2012).  The assays performed were:
1. Larval crawling assay
2. Rapid Iterative Negative Geotaxis Assay.
3. Courtship and Mating Assay.
For the larval crawling assay, larvae were exposed to 5% and 10% ethanol solution.  For the RING assay and Courtship Assay, adult flies were incubated in media containing 15% ethanol and 20% ethanol.

*2.1 Larval crawling assay:*
3$^{rd}$ instar larvae were chosen from a well producing bottle.  Before the treatment with ethanol, they were washed to remove off the excess media covering their body wall musculature.

*2.1.1 Ethanol treatment for larval crawling assay:*
*5% ethanol:*
Individual larvae were placed in a beaker containing 5% ethanol solution and sucrose for 15 minutes.  After 15 minutes, it was transferred onto an agarose coated petriplate.  The petriplate was placed on a graph sheet of 0.2 cm$^2$ grid.  The number of boxes crossed by the larva in 1 minute, over a period of 5 trials was then tabulated.  This was repeated for 4 individual larvae.
*10% ethanol:*
For 10% ethanol test group, new larvae were isolated.  Individual larva was then subjected to 10% ethanol and sucrose solution treatment for 15 minutes.  Once completed it was then transferred onto a new agarose coated petriplate.  The number of grid boxes crossed in 1 minute by the larva over a period of 5 trials was then tabulated.
*Negative control:*
Prior to being placed on the agarose coated petriplate, the larva was allowed to feed in a solution containing 5% sucrose solution for 15 minutes.  After feeding the larva was then transferred onto the petriplate to count the number of grid boxes crossed in 1 minute for over 5 trials.
For each test group, up to 5 larvae were used.  The larvae that were not crawling due to possible injury during collection/washing or treatment were disregarded from the assay.

*2.2 Rapid Iterative Negative Geotaxis Assay:*
For the RING assay, a special apparatus as described in protocol (Nichols, 2012) was constructed.  In this assay, the locomotor behavior of the adult fly is tested.  As the fruit flies are negatively geotaxic by nature, they were made to climb in a scaled tube for a short duration of time.
The concentration of alcohol tested here is 15% and 20% along with the negative control of 0% alcohol.  The adult flies were made to feed on the media containing the percentages of alcohol after starving them for a few hours.  In the assay, the average height climbed by the group of flies in each separate tube was calculated.

After the flies were placed in the respected falcon tubes, they were allowed to acclimatize to the environment for 10 mins.
•       After 10 minutes, the apparatus was tapped 4 times on the surface.  At the 4$^{th}$ tap a timer for 3 seconds was started.
•       At the 3$^{rd}$ second, a picture was taken using the camera.
•       This was repeated for 20 trials with a rest period of 2 minutes for every 4$^{th}$ trial.
The images obtained were then analyzed using ImageJ, and the height climbed by each individual fly in the tube was measured and tabulated.

*2.3 Courtship and Mating Assay:*
Courtship and mating is a complex behavior that involves the recruitment of fine motor movements coupled with the olfactory, visual, and acoustic processes (sensory processing).  Hence it makes for an ideal behavior to assess the effects of alcohol.

The courtship song seen in males is a characteristic combination of movements that involves the following:
1. Orientation [male orients towards female]
2. Tapping [male taps female]
3. Licking [male licks female genitalia]
4. Curling [male curls its abdomen under itself]
5. Copulation attempt [curling activity while attempting to mount female]

*2.3.1 Calculation of courtship index:*

The time at which the behavior occurs is known as 'latency' and the total time engaged in courtship until copulation is calculated as the 'Courtship Index'. Hence the courtship index is calculated as a ratio of:

Time spent in courtship
Total time until copulation

Table 1. Combination of dyads for courtship and mating assay.

| Courting wheel no: | Male fly one fly of concentration : | Female fly one fly of concentration: |
|---|---|---|
| 1 | 0% | 0% |
| 2 | 15% | 0% |
| 3 | 20% | 0% |
| 4 | 15% | 15% |
| 5 | 20% | 15% |
| 6 | 15% | 20% |
| 7 | 15% | 20% |

The frequency observed for wild type is usually between 0.6-0.8 as referenced in (Arthur, 2005).

It is important to separate the male flies from the female flies. Once sorted, they were kept in separate vials for 3 days. They were then starved before being fed on media containing the percentages of alcohol (15%, 20%, and 0%). The combination of dyads for courtship and mating assay is given in Table 1.

In each courting wheel, dyads of the different combination were placed. Each dyad was observed for the courtship song. The courtship *index* was then calculated and tabulated. Different courting wheels were used for each dyad.

## Results

*3.1 Larval crawling assay:*

Considering the developmental stage of the larva and its ability to metabolize the concentration of alcohol, it was decided that a lower dose -5% and a moderate dose-10% were to be tested as higher levels of alcohol prove to be toxic to the larva (Malherbe, 2005). Statistical tests such as standard deviation, standard error of mean (SEM), and a One-way ANOVA were performed.

*3.1.1 Larvae exposed to 0% alcohol: (n = 5)*

Wild type larvae showed normal peristaltic movement. The larvae had a consistent pace of the number of boxes crawled over the 5 trials performed. They showed antics of head lifting, turning, and crawling over the sides of the petri plate.

*3.1.2 Larvae exposed to 5% alcohol: (n = 5)*

The larvae exposed to 5% alcohol for a brief period of time did not have a consistent pace of crawling. They had a sudden burst of activity during the first 2 minutes (in a few it lasted till the first 3 minutes) with a sudden decrease in their locomotion. Moreover, the number of head lifts and turning were much higher in them. After the experiment, these larvae continued to show peristaltic movement in the PBS solution.

*3.1.3 Larvae exposed to 10% alcohol: (n = 7)*

Larvae exposed to 10% alcohol did not have much peristaltic movement (maximum boxes crawled = 4). Moreover, 2 showed no movement over the agarose. The larvae that did show movement were, however,

very slow, with continuous turns made by them.  The larvae stopped very often in their movement and lay still for a few seconds before resuming peristalsis.

*3.1.4 Statistical results:*
        The average or mean of the number of boxes crawled by the larvae exposed to the negative control (0%) was found to be 10.8 boxes, and that of the larvae exposed to 5% alcohol was 7.28 boxes, and 3.58 boxes for the larvae that were exposed to 10% alcohol.  The SEM for the groups 0%, 5%, and 10% were found to be: 0.2315, 0.2497, and 0.12806, respectively (Figure 1).
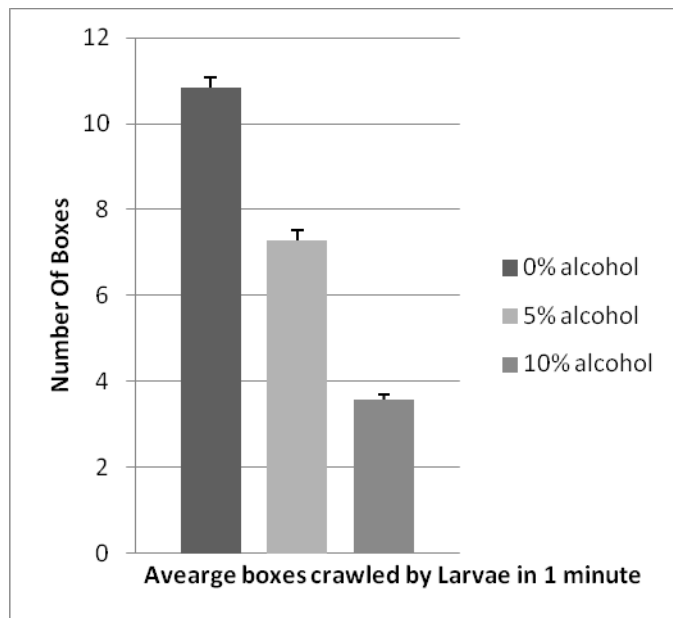


Figure 1.  The graph represents the comparisons of the SEM (error bars) and the average of the number of boxes crawled by 5 larvae in each test group.  The SEM is statistically significant across the test groups, $p < 0.0001$ (one-way ANOVA).

*3.2 Rapid Iterative Negative Geotaxis Assay:*
        During this assay, apart from the average height climbed by the fly, their ability to regain postural control once knocked was also observed. It has been shown that when given a choice between food containing ethanol – 15% to the normal prepared food, flies showed a preference towards ethanol-containing food (Devineni, 2000).
        For each test group, 10 flies were used. A lesser number of flies ensured that the height each fly had climbed was measured.   It also helped in maintaining a consistent sample size during repeated trials.  All images were analyzed using ImageJ.

*3.2.1 Adult flies exposed to 0% alcohol: (n = 10)*
        Young wild type flies had an average climbing height of 4.44 $cm^2$ in a 3 second time period.  The time was set to a 3 second time period to accommodate the maximum height a fly could climb in the given setup. Flies that remained in the bottom were assigned the value of 0.  Over the 10 trials, the flies started showing signs of desensitization towards the 9th and 10th trial.

*3.2.2 Adult flies exposed to 15% alcohol: (n = 10)*
        Adult flies that were exposed to 15% alcohol had shown a biphasic movement towards alcohol.  This was determined by an initial rise in locomotion (determined by climbing height) and a sudden decrease in the ability to climb due to a gradual loss of postural control.  After the first 3 trials, the flies increased the height climbed, and then had a sudden decrease in their ability to climb the tubes.  Their average height climbed was 4.08 $cm^2$.

*3.2.3 Adult flies exposed to 20% alcohol: (n = 10)*
        Adult flies that were exposed to media containing 20% alcohol climbed an average height of 3.37 $cm^2$. Similar to the flies in the test group-15%, the flies in this test group showed an initial increase in their ability to climb at the third trial, after which there was a sudden decrease in their ability to climb.  Moreover during their 7th and 8th trials, the flies did lose postural control leaving most of them at the bottom.  Hence these flies were assigned the value of 0.

*3.2.4 Statistical Results:*

Average heights climbed by the flies of groups 0%, 15%, and 20% were measured as: 4.44 cm$^2$, 4.08 cm$^2$, and 3.37 cm$^2$, respectively. Calculations of standard deviation, standard error of the mean (SEM), and one-way ANOVA were conducted for this assay. The standard deviations for the test groups 0%, 15%, 20% were found to be: 0.6462, 0.3996, and 0.6206, respectively. SEM was calculated and they were calculated as: 0.2043, 0.1263, and 0.1962 for the respective test groups (Figure 2).
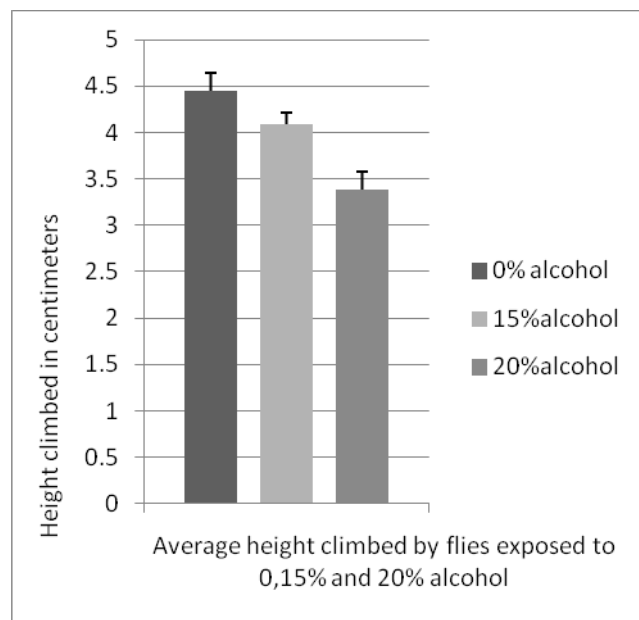


Figure 2. This graph represents the comparisons of the SEM (error bars) and the average height climbed by 10 adult flies in each test group. The SEM is statistically significant across the test groups, p < 0.0009 (one-way ANOVA).

*3.3 Courtship and Mating Assay:*

Courtship index was calculated for the dyads. The results are tabulated in Table 2.

*Dyad observed: Male-0%, Female-0%:*

Wild type female showed consistent signs of application of her tarsi against her partner; the wild type male would circle around the courting wheel to face the female fly. The male fly then showed signs of orientation, which ultimately led to the courtship song. Successful copulation occurred with a courtship index of 0.78.

Table 2. Calculation of courtship index.

| Dyad observed | Courtship Index |
| --- | --- |
| Male - 0%, Female - 0% | 0.78 |
| Male - 15%, Female - 0% | 0.66 |
| Male - 20%, Female - 0% | Copulation not successful |
| Male -15%, Female - 15% | 0.81 |
| Male - 20%, Female - 15% | 0.69 |
| Male - 15%, Female - 20% | 0.40 |
| Male - 20%, Female - 20% | Copulation not successful |

*Dyad observed: Male-15%, Female-0%:*

In this dyad, the wild type female made an attempt to orient towards the male. Consistent grooming followed by orientation of the male fly was observed. After many attempts, successful copulation occurred. The courtship index was calculated: 0.66.

*Dyad observed: Male-20%, Female-0%:*

The male and female were seen to be in close proximity to each other; however, the male found it difficult to stabilize its posture. During the entire courting process it was observed that the male fly lost its stability very often, thus making it quite tedious for it to court the female fly. Male fly eventually made an attempt at courting, but the female fly rejected the male continuously by kicking its tarsi at the male.

*Dyad observed: Male-15%, Female-15%:*

In this dyad, continuous grooming by female was first observed. Rubbing of legs, proboscis extension and abdominal curling are observed in both flies. A brief period of absence in movement by either fly was observed. This was then later followed by tapping and wing extension. The male fly followed the female, until licking took place. Attempts were made at copulation. The courtship index was calculated: 0.81.

*Dyad observed: Male-20%, Female-15%:*

The male made the first attempt by directly tapping the female and then followed it by orientation. Wing extension was prominent in this dyad.  The courtship index was calculated: 0.69.

*Dyad observed: Male-15%, Female-20%:*
        This dyad has a courtship index of 0.40, indicating that the total time spent in courting was very low when compared to the total time until copulation.  Proboscis extension was predominantly seen in this dyad. Towards the end the dyads succeeded in copulation.

*Dyad observed: Male-20%, Female-20%:*
        The male had made an attempt at courting;  however, due to loss of balance and postural control, both flies were seen to be unstable in their movement.  In spite of many attempts, copulation was unsuccessful.

**Discussion and Conclusion**

        Vertebrates and invertebrates have similar activities such as: searching for food, coordinate activity, reproduce with selective mates, and protect themselves from invaders and death.  These behaviors require a synchronized orchestration of sensory inputs and coordination with timely motor outputs.
        The results obtained for the three essays demonstrate that with an increase in the concentration of ethanol, a significant decrease in the assessed behavior- locomotion or courtship is seen.
        In the larval crawling assay, an initial spike of movement was observed with the administration of 5% ethanol;  however, sedating effects were observed gradually with 5% exposure and with the 10% ethanol. Hence we observed a dose-dependent effect of ethanol on the crawling period with higher doses producing a shorter crawling period.  Larval motor circuits are highly developed at the third instar stage (Kohsaka, 2012). The decrease in consecutive motor activity is suggestive that ethanol has an impact on the Ventral Nerve Cord of the *Drosophila*.  It is also known that various neurotransmitters, such as GABA, glutamergic neurons, and adenosine, play a vital role in the recruitment of sensory and motor feedback to produce a specific motor output (Kohsaka, 2012).  Alcohol increases the levels of GABA in the VNC resulting in the characteristic state of sedation and reduced motor output.  This mechanism is evident with the test group of 10% in the larval crawling assay.  Further, the results obtained are in concordance with previous studies (Seggio, 2012).
        Furthermore, the role of alcohol dehydrogenase enzyme and alcohol dehydrogenase (*Adh*) gene is being extensively studied in *Drosophila*, with present data suggesting that the *Adh* gene is known to help in larval tolerance of ethanol.  However, the tolerance obtained in an adult fly does not extend to subsequent larvae formed by it (Malherbe, 2005).
        To further assess the effect of ethanol on the adult motor and sensory circuits, the flies were subjected to the RING assay.  The flies in this assay had shown a consistent biphasic movement.  Mushroom bodies and the antennal lobes play an important role in regulating olfaction.  These neural structures are highly important in the exhibition of the biphasic movement as the initial increase in locomotion is characterized by the smell of ethanol, and the sudden decrease is because of the effect of ethanol on the VNC (Jefferis, 2002).
        The 'biphasic' movement seen in flies is also observed in other animals, such as the rodents (Olivier, 2011), and in humans, where the concentration corresponding to the initial increase in locomotion corresponds to the stage of 'euphoria' in mammals, and the concentration that results in sedation of flies has also shown to cause sedation in humans (Devineni, 2000).  However, it is observed that the 20% ethanol exposure does not cause sedation in the flies.  Instead it causes instability with reference to the posture of the fly.
        The courtship and mating involve interplay of various olfactory, gustatory, and locomotion behaviors. In flies, courtship song and the initiation of mating are exhibited by the male fly.  The female fly, in response to the male, can decide to reject or accept the male.  It was observed that in addition to the characteristic behaviors displayed by the male, the wild-type female and the exposed female flies had shown extension of the proboscis, abdominal curling, and movement toward the male, previously seen in (Kvitsiani, 2006). Moreover, the male flies when exposed to ethanol, took less time to initiate courtship.  Similarly, female flies when exposed to ethanol had shown a lesser rate of rejection.
        It is important to note that sexual behavior, satiation after feeding, and the rewarding effects of particular drugs of use have a common pathway - 'the mesolimbic dopamine pathway' (Steven, 2005).

It is noted that various concentrations of ethanol do produce an effect on the VNC of the fruit fly. In addition to this, the various behavioral parameters observed, and their corresponding results, provide an overview of a few mechanisms by which ethanol acts upon the VNC of the fruit fly.

Further studies that can be conducted in addition to the behavioral assessments include estimation of ethanol concentration in whole fly extracts and development of larval to adult fly after ethanol exposure of the larva.

These tests, along with the behavioral assays can help in elucidating the complex mechanisms responsible for ethanol induced behaviors in flies and also validate the use of the fruit fly as effective model to assess behavioral paradigms for future research.

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### Genetic suppression of *Netrin* adult behavioral defects by *Notch*^lIN-ts1.

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### Abstract

Deletion of both *Netrin* genes (*NetAB*) in adult *Drosophila* leads to behavioral defects that can be suppressed by inhibiting cell death pathways. The *Notch* locus has been shown to play a role in modulating apoptosis, so we tested the ability of a *Notch* temperature sensitive allele (*Notch*^lIN-ts1) to alter *NetAB* phenotypes. Surprisingly, *Notch*^lIN-ts1 was able to suppress *NetAB* locomotor and negative geotaxis defects at the permissive temperature. These results suggest that the *Notch*^lIN-ts1 allele may have subtly impaired function even at permissive temperatures and that *Notch*^lIN-ts1 and *NetAB* mutations display positive epistasis in the adult.

### Introduction

The Netrins are secreted proteins that guide developing axons over short and long distances and are best known for attracting axons to the central nervous system (CNS) midline (reviewed in Lai Wing Sun *et al*., 2011). In *Drosophila*, the two *Netrin* genes (*NetA* and *NetB*) are largely redundant (Brankatschk and Dickson, 2006), although *NetB* has a neurotrophic activity that *NetA* lacks (Newquist *et al*., 2013a). An adult viable stock lacking both *Netrin* genes (*NetAB*^ΔGN) was created by removing a lethal mutation from a *NetAB* chromosome by recombination of the proximal portion of the chromosome. Genetic analysis suggested the existence of a distal mutation near the *white* locus that enhanced viability of the *NetAB* deletion (Newquist *et al*., 2013b). We wished to test the hypothesis that the *Notch* locus might be the gene responsible for the suppressor effect.