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The effects of ethanol on *Drosophila melanogaster* mechanical nociception

Jennifer Le, Mariam Shehata, Isabella Slack
Department of Biology, Rutgers University, Camden, N.J. 08102

Abstract

This study explores the relationship between pain tolerance and alcohol consumption. Alcohol consumption is linked to a decrease in pain sensitivity. Because of this, sufferers of chronic pain can self-medicate with alcohol which may lead to alcoholism. Conversely, sudden withdrawal of alcohol correlates with increased pain sensitivity. When alcoholics try and purge their addiction, they often find themselves more vulnerable to pain and the withdrawal effects are powerful enough to draw them back into alcoholism. Previous studies have shown how alcohol affects the behavior of *Drosophila melanogaster*, a model organism, and demonstrated that in adult flies, their movements become more sluggish. However, the larval stages have not been as extensively studied, especially concerning withdrawal. This study uses a Von Frey hair assay to determine whether there is a significant difference between the reaction of the larvae before and after withdrawal. The results suggest that after a period of steady consumption, the withdrawal of alcohol causes pain sensitivity to increase significantly and to surpass its pre-alcohol exposure level.

Introduction

Alcohol consumption and pain sensitivity are intrinsically linked. Since alcohol is a type of depressant drug, it mollifies the pain; by the same token, the withdrawal of alcohol aggravates the pain (Zale et al., 2015). This inevitably contributes to the addictive nature of alcohol since those who seek it to relieve chronic pain will experience reinforcement of alcohol consumption during withdrawal periods. Recovery from addiction is especially difficult for those in this situation because when they attempt to stop drinking, the pain returns and often seems stronger than before. It is not yet known whether the pain has surpassed its pre-alcohol levels or if it only seems that way because the individual is no longer used to the pain's original intensity, and thus is more vulnerable to it (Zale et al., 2015). Thus, the mechanism behind how alcohol and pain affect one another is worth studying, particularly because the results could inform how best to help those struggling with both alcoholism and chronic pain. *Drosophila melanogaster* is an excellent model for investigating the effects of ethanol consumption and alcohol withdrawal on nociception because several research studies have characterized the behavioral effects of acute/chronic alcohol exposure and pain (Neely et al., 2010). To date, however, no research study has explored the impact of alcohol on pain in *Drosophila*.

While the effects of alcohol on *Drosophila* larvae have not been studied intensely, the same cannot be said about humans. Alcoholism has been associated with the failure to execute tasks or maintain healthy relationships and habits, such as work and school life (Zale et al., 2015). Despite these impaired functions, sufferers of alcoholism continue their routines because of the depressive effects alcohol has on pain. If an alcoholic decides to stop drinking, he or she may face the pain associated with alcohol withdrawal syndrome (Zale et al., 2015), which psychologically reinforces alcohol dependency. Alcohol withdrawal syndrome (AWS) can appear as soon as 6 hours after putting down a glass and symptoms can include headache, nausea, vomiting, hallucinations, fever, and high blood pressure (Saitz et al., 1994).

D. melanogaster, a common type of household fruit flies, share 75% of their pain genes with humans (Milinkeviciute et al., 2012). The conservation of genes are incredibly valuable when studying underlying genetic mechanisms of human experiences. Further, fruit flies develop more rapidly than other model organisms such as mice, so more trials can be run in a relatively short amount of time. A wealth of established knowledge can be found on fruit fly behavior and genetics (Devineni and Heberlein, 2013), which means there is a solid foundation of research on which to build this study. Since much is known about the fruit fly's life cycle, its behavior is well-characterized.

In this study, we used *Drosophila* larvae as the model organism. When exposed to pain, the larvae demonstrated a 360° lateral rolling motion (Tracey et al., 2003) which can be quantified. Third instar larvae, the most advanced stage, will be used because they are large enough to be manipulated with relative ease, and because they are developed enough to perform the rolling motion. The rolling behavior, which we seek to quantify, does not persist into adulthood.

It must be noted that while the study ultimately seeks to aid sufferers of chronic pain, the rolling behavior is an indicator of nociception. Nociception is the neurological signal associated with pain, rather than the painful sensation itself.

The signal travels through the organism's spinal cord when stimulated by pain and causes a behavioral response., leading to the subjective experience of pain. The pain will be induced using Von Frey hairs.

Von Frey hairs (VFH) are thin filaments that are used to apply a specific amount of force. They are used in pain studies such as the analyses of allodynia and hyperalgesia (Jensen and Finnerup, 2014). Respectively, the terms are a pain response to a non-painful stimulus and an enhanced pain response to an already painful stimulus. VFHs apply a specific amount of mechanical force with each application and elicit a rolling behavior from *Drosophila* larvae when the force is applied. The VFH applies the maximum amount of force when it buckles under pressure, and the force applied is inversely proportional to the length of the hair (Zhong et al., 2010). Therefore, the shorter the hair, the greater the force applied to the larva. Since the length of the hair is integral to the response rate, the length must be adjusted to elicit a response 20%-40% of the time (Deuis et al., 2017). Although VFHs are more known for their properties in experiments involving mice, the same properties can be applied to *Drosophila* larvae. There is a possibility that some of the larvae will be overstimulated and die but that problem can easily be remedied by breeding plenty of animals.

Along with the threat of overstimulation, there is a possibility that ethanol can have adverse effects on the larvae. Fruit flies lay their eggs in environments with ethanol concentrations as high as 7% (Fry, 2014), so *Drosophila* eggs and larvae are less adversely affected by the typically harmful substance (Devineni and Heberlein, 2013) but higher concentrations can have adverse or even lethal effects due to the high energy cost of combating the ethanol. One such effect is stunted growth (Castañeda and Nespolo, 2013). These obstacles must be overcome for the study to be conducted successfully and have been ameliorated in the following methods.

As previously mentioned, the threat of overstimulation can be remedied by breeding many animals such that the population can be split into two sets in which each set experiences a VFH treatment once. The effects of ethanol on larvae development can be mollified by inserting the larvae into the supplemented media after hatching in the non-supplemented media. This will decrease the growth stunting effect of the ethanol and will lead to the development of more third instar larvae. Both methods have been implemented in the procedure. This study seeks to determine how the exposure and withdrawal of ethanol in fruit fly larvae affect their mechanosensory nociceptor sensitivity. It is hypothesized that ethanol exposure decreases nociception while ethanol withdrawal increases nociception in *Drosophila melanogaster* larvae.

Materials & Methods

Fly rearing

Wild-type flies were graciously given by Dr. Lee's lab. The flies were allowed to breed in a petri dish containing a grape juice agar and a yeast paste, topped with an embryo collection cage. Two embryo collection cages were made to rear two sets of flies: one that would be exposed to ethanol, put through a VFH treatment, and discarded, and one that would be exposed to ethanol, removed from ethanol for a withdrawal period, put through a VFH treatment, and then discarded (see Fig. 1). The collection cages were placed in an incubator set for a 12 hour day/night cycle at 22.5° C (Pulver and Berni, 2012). A 600mL beaker full of water was placed under the collection cages to ensure that neither the agar nor the yeast paste would dry out.

The petri dishes were supplied with as many flies as would fit without having to walk on one another. Then the flies were allowed two days in the chambers; one to acclimate to their new living conditions, and one to lay their eggs. After two days, the agar and eggs in the chambers were transferred to a 0% ethanol vial. Once the larvae had hatched and became mobile, they were transferred to the appropriate treatment vials (see Ethanol treatment). Approximately 5 days were needed between hatching and the first VFH treatment to allow the larvae to reach the third instar stage (Ong et al., 2015).

Ethanol treatment (acute and withdrawal)

The larvae from the collection chambers were divided equally into eight vials, two of each concentration. Four different concentrations were used: 0%, 5%, 10%, and 20%. The ethanol was mixed within the cornmeal media, on a v/v basis. The 0% concentration served as the control for this experiment and was made simply by not supplementing a vial of media with ethanol. For the withdrawal portion, the second group of larvae went through a 24-hour withdrawal period, feeding on media with a 0% ethanol concentration.

VFH treatment for mechanical nociception

Petri dishes filled halfway with plain agar for moisture will be used as a platform to perform this assay. The first treatment will be administered approximately 30 minutes after the day cycle begins in the incubator. Using a VFH of a length of 0.015 m (1.5 cm), the larvae will be subjected to a poke of 1.79 g, ten times each, regardless of whether the poke elicited a response or not. The positive responses, the ones that elicited the rolling behavior, will be taken as a percentage of the total amount of applications. When this treatment is done, all the subjected larvae will be discarded.

After the withdrawal period, there will be a second treatment using the second group of larvae. The same VFH length will be used, and the treatment will be administered at the same time of day as the first treatment.

Statistical Analysis

All the larvae used were from the same cohort, split into two groups: one that will be stimulated with the VFH assay immediately after exposure, and one that will be stimulated after withdrawal. For Figure 1, a one-way ANOVA was conducted to test the relationship between the percent response and the applied force. For Figure 3, the student's t-test was used to compare the results of the response of the larvae before and after withdrawal. A one-way ANOVA was used to test whether the ethanol concentrations influenced the response rates. A two-way ANOVA was conducted to see if the interaction between the two terms, the concentrations of ethanol and whether withdrawal affected the response rates, were significant.

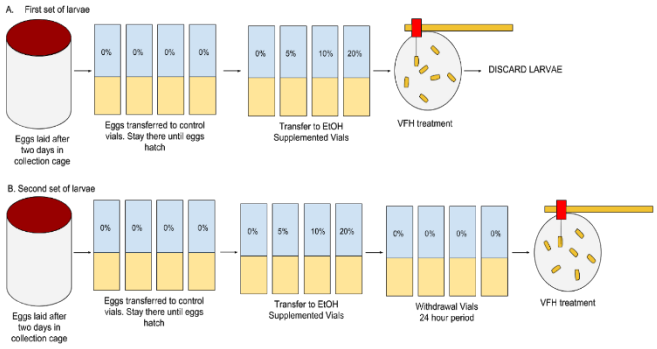

Figure 1. The experimental procedure as described in the materials and methods. The cylinder on the far left represents the embryo chamber from which the eggs were hatched. The four rectangles represent the vials with fly food which the larvae lived and fed on. The drawings on the far right represent the petri dishes with larvae and the apparatus touching the one of the larvae is a VFH filament attached with red tape to a popsicle stick.

Results

Von Frey hair Assay for Mechanical Nociception

To determine the correct VFH length, tests were run where VFHs of uniform width and various lengths were used to perform assays on larvae that were not exposed to ethanol, with the goal of finding the length at which a 50% response rate was recorded. The purpose of this was to obtain a force that produced a response rate that was high enough to decrease with ethanol exposure and low enough to increase with ethanol withdrawal. The data reflects the larvae being successfully poked with the VFH at a 90° angle, five times. The percent response was taken as the amount of positive responses divided by the total pokes. It was found that the higher force of a shorter VFH elicited more responses than the lower force longer lengths (t-test, $p = 0.001788274$, $p < 0.05$, $n = 5$). Since a length of 1.5 cm was used to establish a 50% response, applying a force of 1.79 g, we used the same hair for all subsequent experiments.

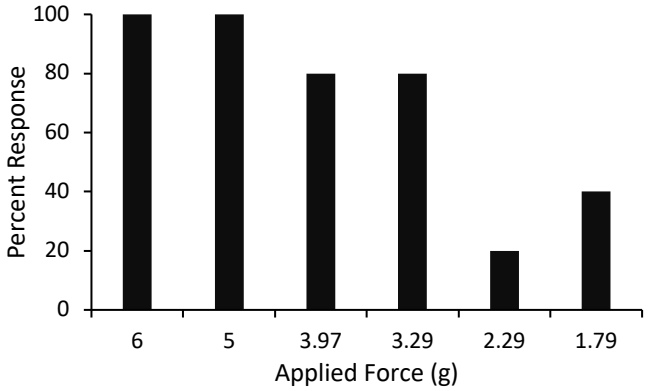

Figure 2: Von Frey hair Assay for Mechanical Nociception. The data was obtained by stimulating the larvae five times, with the VFH angled at 90°. The percent responses were taken as the number of positive responses out of the total responses, regardless of whether the larvae rolled.

Von Frey hair Assay Before and After Withdrawal

The first exposure data was obtained through taking the larvae out of their respective vials and applying the VFH 10 times and taking the fraction of the positive responses out of the total pokes. The after-withdrawal data was obtained by first moving the larvae out of their ethanol supplemented vials, placing them into the withdrawal vials, and taking them out for testing after 24 hours. The testing was done in the same manner as the first exposure. Before withdrawal, the percent responses were not significant (ANOVA, $p = 0.209945$, $p > 0.05$, $n = 61$). The 20% vial's larvae failed to respond at all. Between only the withdrawal data, the percent responses were not significant (ANOVA, $p = 0.066186$, $p > 0.05$, $n = 43$). The difference in responses before and after, however, were significant (t-test, $p < 0.05$) for the 5% and 10 vials. Only the 0% vial and the 20% vial did not see a significant difference (t-test, $p > 0.05$). The interaction between the concentrations and the before and after withdrawal terms were non-significant (two-way ANOVA, $p > 0.05$), and withdrawal had a significant effect on the data (two-way ANOVA, $p < 0.05$) whereas the concentrations did not.

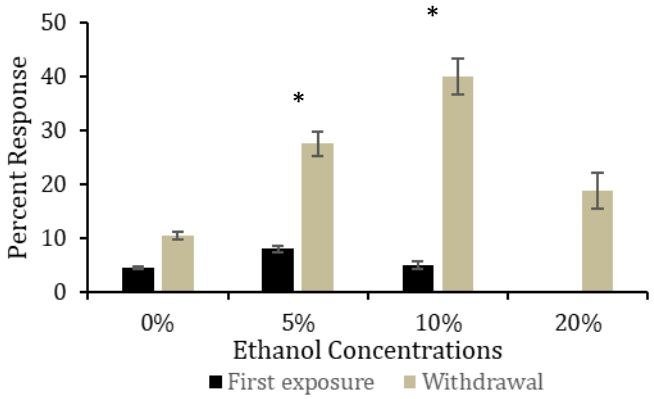

Figure 3. Percent responses before and after withdrawal. Within each concentration, the first exposure data are on the left and the withdrawal data are on the right. For 20%, there is only withdrawal data. For the first exposure data, in ascending order of concentrations, the sample sizes are: n = 31, n = 17, n = 16, and n = 5. For the withdrawal data, in ascending order of concentrations, the sample sizes are: n = 20, n = 12, n = 3, and n = 8. The asterisk (*) indicate groups that have a significant difference of $p < 0.05$.

Discussion

The purpose of this study was to determine what effect, if any, ethanol consumption had on nociception in *D. melanogaster*. The data collected from animals in the first group, those who had been exposed to ethanol at various concentrations without undergoing withdrawal, showed no significant difference in nociceptor activity with respect to concentration ($p > 0.05$). Figure 3 shows that the larvae in the 5% vial rolled more than the control in the first exposure group, contradicting the hypothesis that ethanol inhibits nociception. This could be explained by the fact that in the wild, the larvae live and feed within an environment that is naturally rich in ethanol (Devineni and Heberlein, 2013). The same is not true of humans, so the possibility of ethanol inhibiting nociception in humans cannot be dismissed. Furthermore, the first exposure responses were lower than expected: under 10% across all concentrations. Before conducting the experiment, proper testing was done to determine an optimal VFH length, so the treatment can elicit a 40% response rate out of 10 pokes. Figure 2 depicts the results of this test and during the actual experiment, the response rates were unexpectedly low compared to what Figure 2 suggested. Genetic variation could explain the inert nature of the larvae used in the experiment, as the larvae were all from the same cohort. Additionally, the sample size was low, which makes it more likely to deviate from the average response rate. The larvae in the 20% exposure vial did not respond at all. In previous studies, larvae growing in an ethanol-supplemented environment have taken longer to develop in comparison with ones raised in an ethanol-free environment (Castañeda and Nespolo, 2013).

After the initial treatment, all the larvae used were disposed. Larvae in the second set of vials were moved to control vials for 24 hours to simulate withdrawal. The percent responses after withdrawal significantly increased ($p < 0.05$), except for the 20% vial ($p = 0.08$). The larvae in the 10% vial had the sharpest increase in response but this could be due to the small sample size of n = 3. Unexpectedly, the 20% group had the least change in responses. The concentration may have been too strong and could have sedated the larvae such that they were unable to react (Scholz et al., 2000), as can occur in humans. Lowering the concentration could increase the response rate. The differences between withdrawal response rates were insignificant ($p = 0.06$), which suggests that the concentrations had no effect. For the 0% vial, before and after withdrawal, we did not expect a change since the larvae's conditions were not altered in any way. The data in Figure 3 reflects this ($p > 0.05$).

From the results of this experiment, one can conclude that *Drosophila* larvae are subject to the negative effects of alcohol withdrawal with response rates increasing across all concentrations. However, increasing the concentrations did not significantly chance responses before withdrawal. Due to the low replication and low sample sizes, a stronger conclusion cannot be made. This experiment has also refined the VFH assay for larvae pain sensitivity, though the hair was applied manually so there could be variations in how it was applied. In future studies, the alcohol concentrations should be adjusted to ensure that the larvae are able to develop, to examine whether being exposed to the ethanol has long-term effects.

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