JOURNAL OF
EMERGING INVESTIGATORS

Article

# Substance abuse transmission-impact of parental exposure to nicotine/alcohol on planaria offspring

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## SUMMARY

**There is an unprecedented global mental health crisis, and substance abuse among youth is a growing issue. According to the Centers for Disease Control and Prevention, 40 million adults in the United States smoke cigarettes, and 4.7 million children have used at least one tobacco product. Abuse of prescription medicines is the cause the death for about 115 Americans daily. While substance abuse tendencies seem transmissible from one generation to next, human studies are challenging due to the long duration and confounding socio-economic variables. Recent Food and Drug Administration guidance requires abuse liability testing in animals and humans for neuro-active medicines. However, there is no requirement for testing intergenerational transfer of abuse potential. Based on the similarities in nervous system development between mammalian embryos and regenerating planaria, we hypothesized that regenerating offspring of brown planaria parents exposed to substances of abuse (nicotine or ethanol) will demonstrate conditioned place preference similar to parent planaria, making them an alternative model for testing intergenerational transfer of substance abuse potential. Parent brown planaria exposed to nicotine or ethanol showed CPP in a concentration and time-dependent manner. We also demonstrated that regenerating brown planaria offspring are more sensitive than parents to CPP by nicotine or ethanol. In addition, exposure of parent planaria to nicotine or alcohol resulted in CPP in the regenerated offspring, without direct exposure to nicotine or ethanol during regeneration. Based on these results, regenerating brown planaria is a promising alternative model for testing intergenerational transfer of substance abuse potential.**

## INTRODUCTION

Substance abuse has been a universal problem affecting individuals irrespective of their of age, gender, race, class, or societal standing (1, 2). Chemical abuse is associated with serious physical and mental health issues, and has a significant financial impact on families and society (3). Youth substance abuse is a growing issue and a recent report by the National Center for Drug Abuse Statistics revealed shocking trends in substance abuse over the past two decades, including a 61% increase in drug use among 8$^{th}$ graders from 2016-2020, alcohol abuse among 62% teens in 12th grade and a significant increase in daily smoking in early adulthood from 2002 to 2018 (4). The Substance Abuse and Mental Health Services Administration reported that the fastest growing substance abuse problem is not cocaine, heroin, or methamphetamines, but the abuse of prescription drugs such as opioids, stimulants, and antidepressants (5). The number of medicines approved for neurologic and psychiatric disorders increased significantly since the early 2000s and many neuroactive drugs could have an increased potential for abuse (6). Drugs and other substances with abuse potential are not only injurious to individuals who use them, but pose a risk to their children (7–9). While substance abuse tendencies seem to be transmissible from one generation to next in humans, confirming this is challenging due to the long duration needed for testing, and the confounding socio-economic variables involved (10). Since mammalian animal studies are costly, take long time, and use a lot of mammals, scientists are searching for alternative toxicity testing models for safety assessment of chemicals and medicines (11).

Conditioned place preference (CPP) is a form of Pavlovian conditioning used to measure the motivational or reward effects of objects or experiences and is a standard endpoint in abuse liability studies in mammals (12). CPP is the preference of an organism to an undesirable area that has been associated with a reward (such as the drug), indicating how much the organism likes the reward and identify if a substance is considered a “reward” or addictive (13).

Freshwater planaria, an alternative animal species that is increasingly used for toxicity testing, have a centralized nervous system with a wide range of cell types, behavioral responses, complex mechanisms of regeneration, and multiple neurotransmitters involved in risk/reward pathways (14). In addition, planaria exhibit mammalian-like behavior responses, including environmental place conditioning (15). Planaria normally prefer dark environment, but will choose well-lit environments if light is paired with a reward, which makes them a suitable species to test CPP using light (16). Planaria can create offspring by both sexual and asexual reproduction, and planarian regeneration shares similarities with the development of mammalian embryos, especially in the development of the nervous system (17, 18). The remarkable ability of planaria to regenerate a new head within two weeks after amputation, makes it a prospective alternative model to test intergenerational transmission of substance abuse potential to regenerated offspring (19). Previous studies showed that short-term exposure of planaria to substances such as cocaine, glutamate, or methamphetamine resulted in sensitization behavior including hyperkinesia (C-curling), changes in motility, and stereotypical activity (20). This study is the first to assess the suitability of brown planaria as a relevant alternative model to test chronic substance abuse and the intergenerational transmission of substance abuse potential.

https://doi.org/10.59720/23-014

Nicotine and ethanol were considered as suitable reagents to test the transmission of substance abuse behavior from parent planaria to regenerated offspring because both nicotine and alcohol are known to have abuse liability and have been part of human societies for multiple centuries, with an established link between parental smoking and alcoholism to dependence in children (7, 8). The purpose of this research was to test whether brown planaria (*Dugesia tigrine*) will be a good model to test the intergenerational transmission of substance abuse potential. The main hypothesis was that directly exposing regenerating planaria to known substances of abuse such as nicotine or alcohol during development will increase their sensitivity to conditioned place preference (CPP). We also hypothesized that conditioning of parent planaria with nicotine or alcohol will cause conditioned behavior in newly regenerated planaria (without direct exposure to these substances during development), and regenerating planaria is more sensitive to nicotine or ethanol compared to parent planaria. Results from our studies demonstrated that parent brown planaria exposed to nicotine or ethanol showed CPP in a concentration and time-dependent manner. We also discovered that regenerating brown planaria offspring are more sensitive than parents to CPP by nicotine or ethanol. Finally, we found that exposure of parent planaria to nicotine or alcohol resulted in CPP in the regenerated offspring, without direct exposure to nicotine or ethanol during regeneration.

## RESULTS

### Prolonged daily exposure to nicotine or ethanol induced tolerance in parent planaria

Tolerance is the ability to endure the effects of a drug without any adverse reaction and was assessed by the loss of "C" like curling of planaria when exposed to nicotine or ethanol. To be relevant to the human substance abuse scenario, the lowest nicotine concentration (300 nM) tested in the definitive experiment was similar to the average plasma levels of nicotine in smokers (~280 nM) (21). The lowest level of ethanol (0.05%) used in the definitive experiment was the legal blood alcohol limit in many countries in Europe and Asia (22). The highest concentration of nicotine (100 µM) and ethanol (1%) was selected based on results from a pilot experiment (data not shown). Parent planaria were exposed to varying concentrations of nicotine, ethanol or their respective vehicle controls, for an hour per day in the presence of light, for 10 days (**Figure 1**). C-curling was observed from day 1 to day 8 in planaria exposed to 100 µM nicotine, and from day 1 to day 5 in the 3 µM nicotine group (**Figure 2A**). Among the parent planaria exposed to ethanol, C-curling was observed from day 1 to day 6 in the 1% ethanol group, and from day 1 to day 5 in the 0.2% ethanol group (**Figure 2B**). There was no C-curling observed in parent planaria exposed to glycerin (1:6000 dilution corresponding to the glycerin concentration at 100 µM nicotine), spring water, 0.3 µM nicotine or 0.05% ethanol during days 1 through 10.

Among the parent planaria exposed to nicotine, the mean

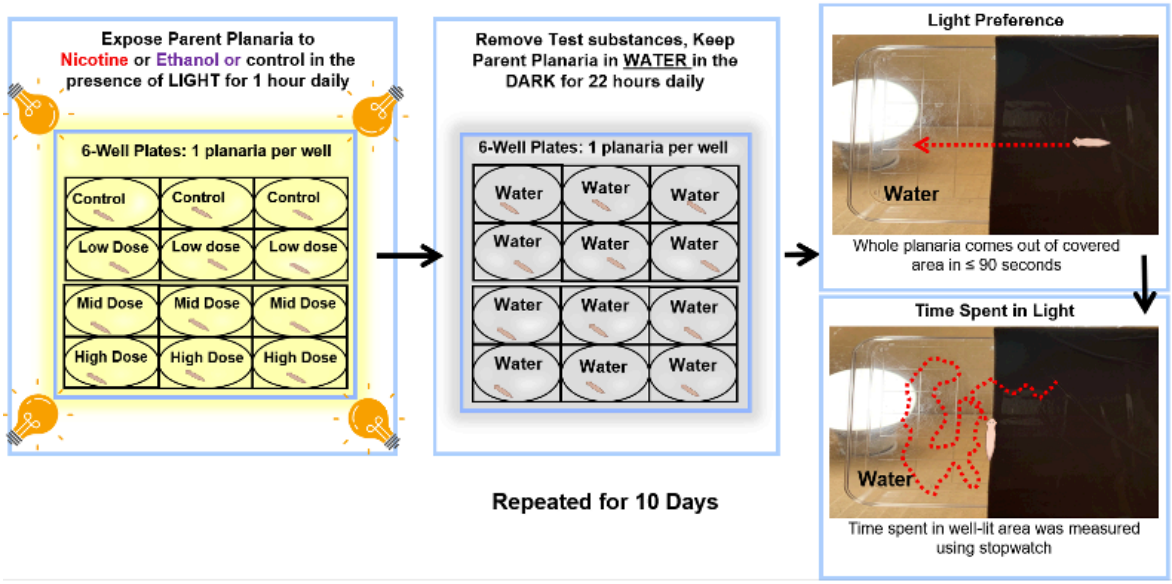


**Figure 1: Phase 1 - Experiment design for testing conditioned place preference of parent brown planaria to establish adult planaria as a substance abuse testing model.** Parent brown planaria (n=6 per group for controls and low dose groups; n=3 per group for mid and high dose groups) were exposed to light in the presence of 3 concentrations of nicotine or ethanol and their corresponding vehicle controls (glycerin and spring water). For nicotine treated group, the control, low dose, mid dose and high dose were 1:1000 dilution of glycerin, 0.3, 3 and 100 µM nicotine, respectively. For ethanol treated groups, the control, low dose, mid dose and high dose were spring water, 0.05%, 0.2% and 1.0% ethanol, respectively. After 1 hour of light exposure, the test substances were removed, planaria were washed 3 times and the planaria were maintained in spring water in a dark environment. After 22 hours, conditioned place preference of parent planaria were evaluated by light preference and time spent in light, daily for 10 days. Light preference was evaluated by placing individual planaria towards one end of a square integrid petri dish containing spring water on the light box. The half of the petri dish with planaria was inserted into a small box to create a dark space. An observation of light preference was made if the whole planaria moved to the well-lit region within 90 seconds. Time spent in light was evaluated using the same half-covered petri dish and spring water. Planaria were observed for 90 seconds after placing the planaria at the intersection of dark and well-lit regions. The time planaria spent in the well-lit region of the petri dish was measured (in seconds) using a stopwatch.

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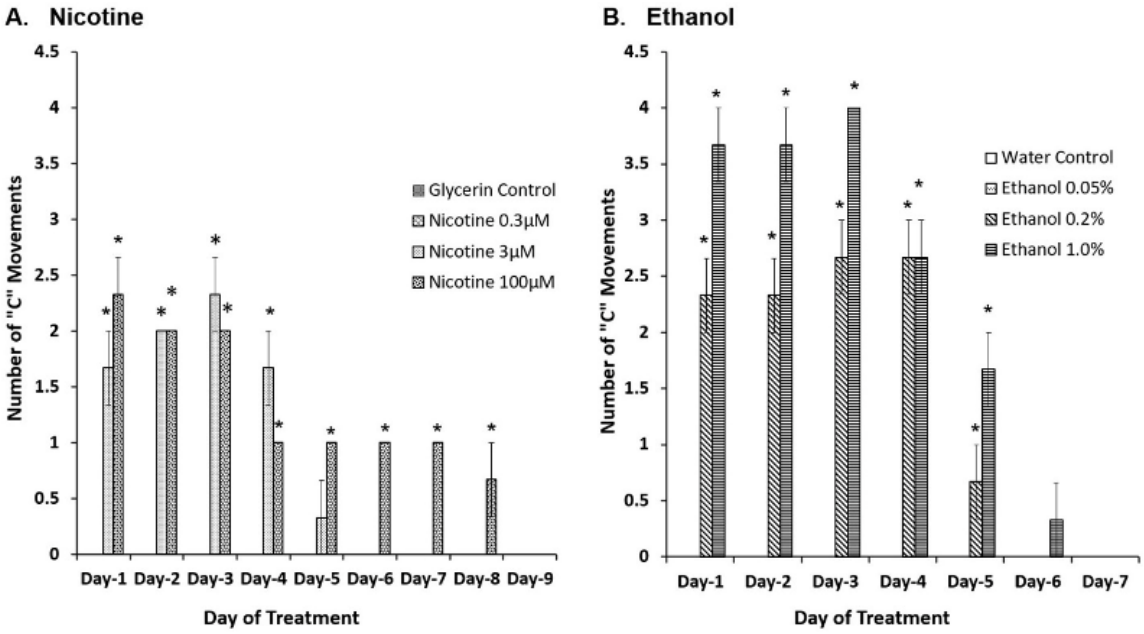


**Figure 2. Effect of nicotine or ethanol on C-shaped hyperkinesia (C-curls) of parent planaria.** Bar graph depicting the effect of chronic nicotine (A) or ethanol (B) exposure in inducing tolerance in parent planaria, showing the mean number of C curls (± standard error) made by planaria on different days of test article treatment. Tolerance was assessed by absence of the "C" like curling of planaria when exposed to nicotine or ethanol. N=6 per group for water control, glycerin control, 0.3µM nicotine and 0.05% ethanol groups, N=3 per group for 3 and 100 µM nicotine groups, and 0.2% and 1% ethanol groups; * P < 0.001.

number of C-curls/planaria was the highest (2.33/planarian) on day 1 at 100 µM concentration, followed by a gradual reduction to 0 by day 9. At 3 µM nicotine concentration, the mean number of C-curls/planaria were 1.67 on day 1, 2.33 on day 3, and 0 by day 6 (**Figure 2A**). The mean number of C-curling/planaria in the 1% ethanol group were 3.67 on day 1, reaching the highest (4 C curls/planarian) on day 3 and gradually declining to 0 by day 7. The trend was similar in the 0.2% ethanol group with the mean number of 2.33 C curls/planarian on day 1, progressing to 2.67 C curls/planaria on day 3, followed by a reduction to 0 C-curls by day 6 (**Figure 2B**). Based on the complete absence of C-curling in the vehicle control groups and statistical analyses, this C-curling was considered statistically significant (t-test, $p \leq 0.001$) at ≥ 3 µM nicotine and at ≥ 0.2% ethanol (**Figure 2**). While parent planaria exposed to ethanol showed higher mean number of C-curls/planarian compared to nicotine until day 5, C-curls persisted for the longest duration (8 days) in planaria exposed to 100 µM nicotine. Planaria exposed to higher concentrations of nicotine or ethanol took longer to develop tolerance compared to planaria exposed to lower concentrations, suggesting that tolerance development was dependent on the concentration of these substances (**Figure 2**). Overall, planaria developed tolerance to high concentrations of nicotine by 6-9 days and to ethanol by 6-7 days, demonstrating a concentration and time-dependent effect (**Figure 2**). Gradual reduction in the number of C-curls with continued daily exposure of ethanol or nicotine is consistent with development of tolerance to these substances, and shares similarities with the development of tolerance or addiction in humans. This data provided the rationale for using 10 days of tolerance in the parent planaria, before amputation on day 11.

## Parent planaria exposed to nicotine or ethanol showed conditioned place preference

CPP of parent planaria was evaluated in phase 1, ~22 hours after light conditioning in the presence of nicotine or ethanol, daily, for 10 days (**Figure 1**). For parent brown planaria exposed to varying concentrations of nicotine or ethanol, light preference (**Figure 3**) and time spent in a well-lit environment (**Figure 4**) were evaluated, to investigate the CPP of planaria in response to these substances of known abuse potential. Parent planaria exposed to nicotine or ethanol showed preference for light in a time- and concentration-dependent manner at or above 3 µM nicotine (**Figure 3A**) or 0.2% ethanol (**Figure 3B**). Specifically, 33% of parent planaria displayed light preference by day 2 and day 5 in the 100 and 3 µM nicotine exposed groups, respectively. All the parent planaria at 100 and 3 µM nicotine showed light preference from day 3 and day 6 onwards, respectively (**Figure 3A**). Among parent planaria exposed to ethanol, 33%, 67% and 100% planaria showed light preference on days 3, 4 and 5 respectively. In the 0.2% ethanol group, 67% displayed light preference on day 6 and 100% showed light preference from day 7 onwards (**Figure 3B**). Parent planaria exposed to glycerin, spring water, 0.3 µM nicotine or 0.05% ethanol did not display light preference on any of the 10 days of testing. These results demonstrate that adult planaria conditioned to light in the presence of nicotine or ethanol displayed light preference in a concentration and time dependent manner.

Statistically significant increases (t-Test $p \leq 0.001$) in the time spent in well-lit environment was noted by day 3 and day 4 in the 100 µM nicotine (**Figure 4A**) and 1% ethanol groups (**Figure 4B**), respectively. Planaria in the 100 µM nicotine group spent an average of 63.3 to 66.0 seconds in light from days 6 through 10, compared to 15.3 to 16.0 seconds by

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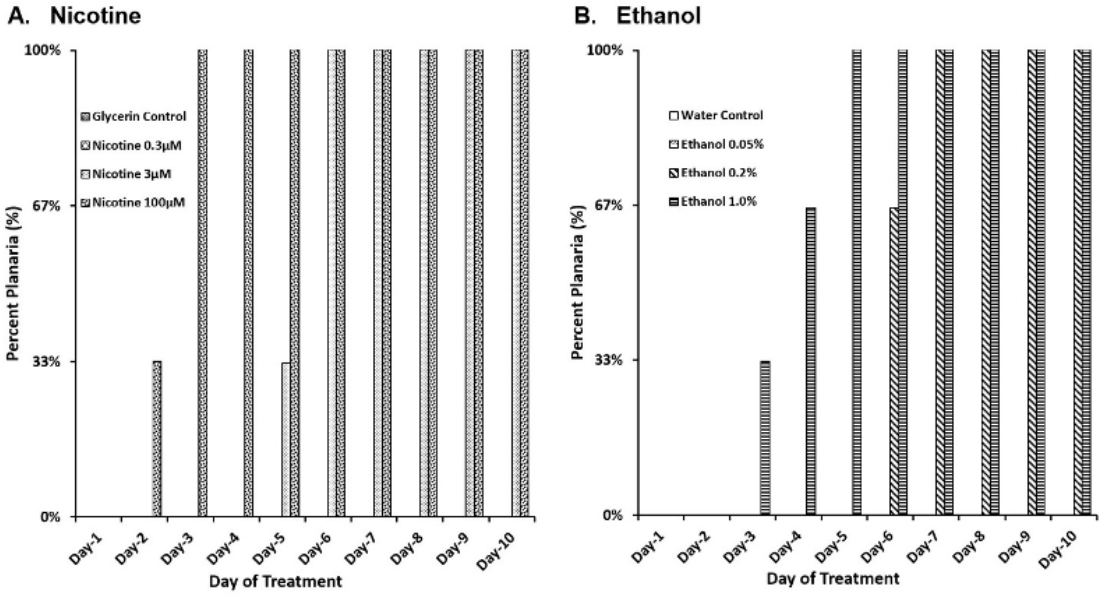


**Figure 3. Effect of nicotine or ethanol on light preference by parent planaria.** Bar graph depicting the effect of nicotine (A) or ethanol (B) on light preference by parent planaria, showing the percentage of planaria showing light preference on different days of test article treatment. N=6 per group for water control, glycerin control, 0.3 µM nicotine and 0.05% ethanol groups, N=3 per group for 3 and 100 µM nicotine groups, and 0.2% and 1% ethanol groups.

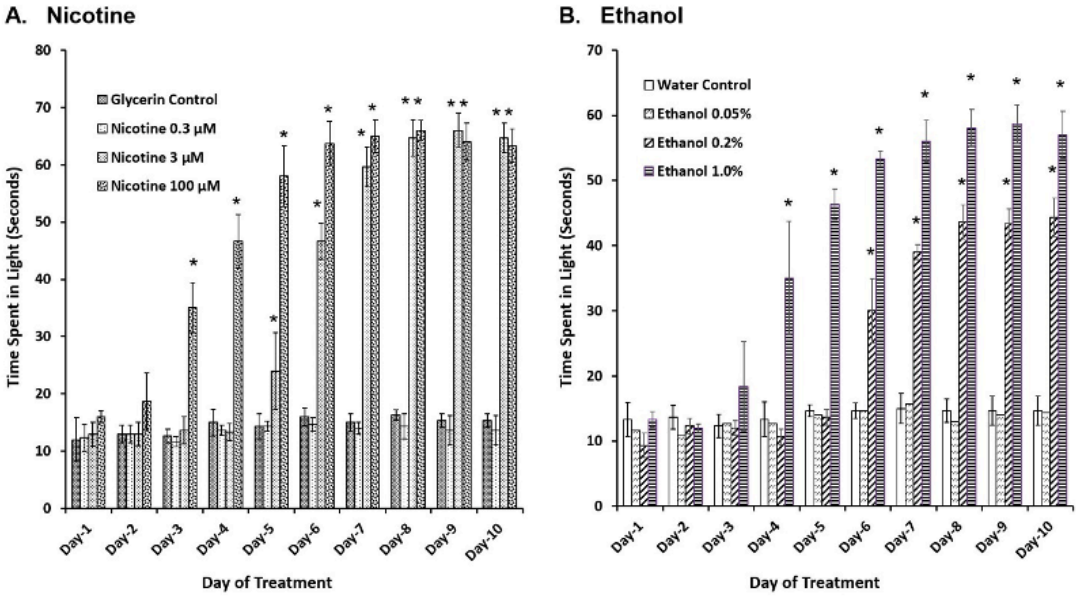


**Figure 4. Effect of nicotine or ethanol on time spent in light by parent planaria.** Bar graph depicting the effect of nicotine (A) or ethanol (B) on time spent in light by parent planaria, showing the mean number of seconds (± standard error) spent in light on different days of test article treatment. N=6 per group for water control, glycerin control, 0.3 µM nicotine and 0.05% ethanol groups, N=3 per group for 3 and 100 µM nicotine groups, and 0.2% and 1% ethanol groups.

planaria in the glycerin control group (**Figure 4A**). Similarly, planaria in the 1% ethanol group spent 56.0 to 58.7 seconds in the light from days 7 through 10, compared to ~15 seconds by planaria in the spring water control group (**Figure 4B**). Statistically significant increases (t-test $p \leq 0.001$) in time spent in light was also observed at the 3 µM concentration of nicotine and 0.2% ethanol, with planaria exposed to nicotine spending 64.7 to 66 seconds by day 6 and planaria exposed to ethanol spending 43.7 to 44.3 seconds from days 8 through 10 (**Figure 4**). There were no statistically significant differences in the time spent in light among the parent planaria exposed to glycerin, spring water, 0.3 µM nicotine or 0.05% ethanol during days 1 through 10. These results demonstrate that parent planaria conditioned to light in the presence of nicotine or ethanol, spent a statistically significantly longer time in a well-lit environment in a concentration and time dependent manner.

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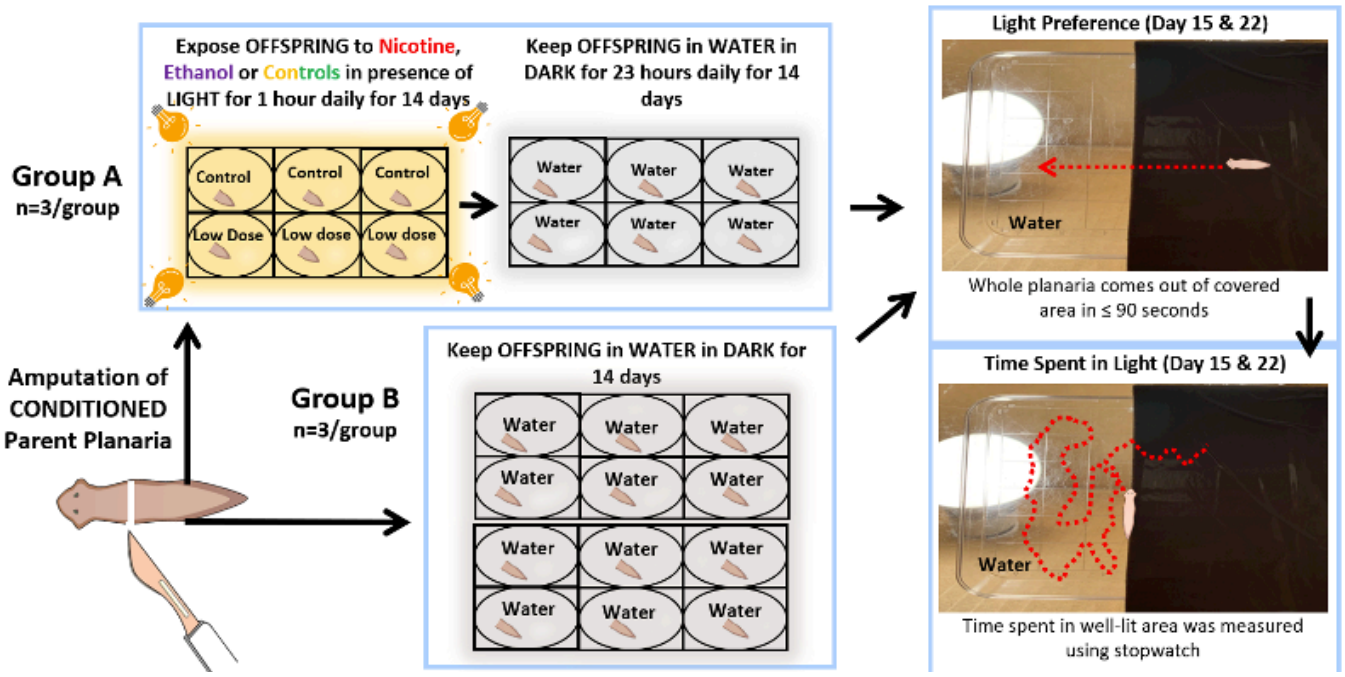


**Figure 5. Phase 2 - Experiment design for testing conditioned place preference of regenerated planarian offspring.** Head of parent planaria were amputated after 10 days of conditioned place preference and the tail sections were allowed to regenerate a new head for 14 days. Tails from a subset of control and low dose groups (Group A; n=3 per group) were conditioned to light in the presence 0.3μM nicotine or 0.05% ethanol, similar to parent planaria (1-hour daily exposure to light in the presence of nicotine, ethanol or corresponding vehicles followed by washing 3 times in spring water, maintained in water and kept in a dark environment for 23 hours) for 14 days. Tails from control and all dose groups (Group B; n=3 per groups) were maintained in water and kept in a dark environment for 14 days. Conditioned place preference of regenerated offspring planaria were evaluated by assessing light preference and time spent in light, on post amputation day 15 and day 22.

### Regenerated offspring of conditioned parent planaria displayed CPP without direct exposure to nicotine or ethanol

During phase 2, tails of amputated parent planaria conditioned to light in the presence of varying concentrations of nicotine or ethanol were allowed to regenerate in spring water for 14 days without any exposure to these substances during development (**Figure 5, Group B**). The CPP of these regenerated offspring was evaluated by their light preference and the time spent in light on day 15 and day 22 post amputation. All the regenerated offspring of parent planaria exposed to 100 μM nicotine continued to display light preference on post amputation day 15 and day 22. In addition, 33% of the regenerated offspring at 3 μM nicotine and 67% of the regenerated offspring at 1% ethanol showed light preference on post amputation day 15 and day 22 (**Figure 6A**). On both post-amputation day 15 and day 22, there was statistically significant (t-test $p \leq 0.001$) increase in the time spent in a well-lit environment by regenerated offspring of parent planaria conditioned to light in the presence of 100 μM nicotine or 1% ethanol, compared to respective vehicle controls (**Figure 6B**). These results demonstrated that nicotine or ethanol induced CPP is transferred from parent to regenerated planarian offspring, even without direct exposure to these known substances of abuse during regeneration.

### Direct exposure of regenerating brown planaria offspring to low concentrations of nicotine or ethanol during development resulted in CPP

During phase 2, tails of amputated parent planaria conditioned to light in the presence of varying concentrations of nicotine or ethanol were allowed to regenerate in spring water for 14 days. The effect of nicotine or ethanol exposure on regenerating planaria offspring (**Figure 5, Group A**) was evaluated by continued light conditioning of amputated tails in the presence of 0.3 μM nicotine or 0.05% ethanol for one hour daily during the 14 days of head regeneration. The light conditioning employed similar methods as used for parent planaria. Glycerin (1:6000 dilution) and spring water were used as vehicle controls corresponding to nicotine and ethanol. Light preference and time spent in a well-lit environment were evaluated to understand CPP on days 15 and 22 post amputation. The head was completely regenerated in control and test item treated groups by 14 days post amputation and digital microscopic evaluation did not reveal any apparent defects in the regenerated offspring. All the regenerated planaria offspring exposed to 0.3 μM nicotine and 67% of the regenerated offspring exposed to 0.05% ethanol showed light preference on post amputation day 15 and day 22 (**Figure 7A**). Regenerated planaria offspring exposed to nicotine or ethanol during development spent 32-34 seconds in a well-lit environment compared to 13-18 seconds by planaria in the control groups, demonstrating a statistically significant increase (t-test $p \leq 0.001$) in the time spent in light by the regenerated offspring exposed to these substances (**Figure 7B**). The concentrations of nicotine (0.3 μM) and ethanol (0.05%) that caused CPP in regenerated offspring were lower than the lowest concentrations that caused conditioning in parent planaria, indicating that parental exposure to nicotine or ethanol led to an increased sensitivity to CPP in regenerating planaria.

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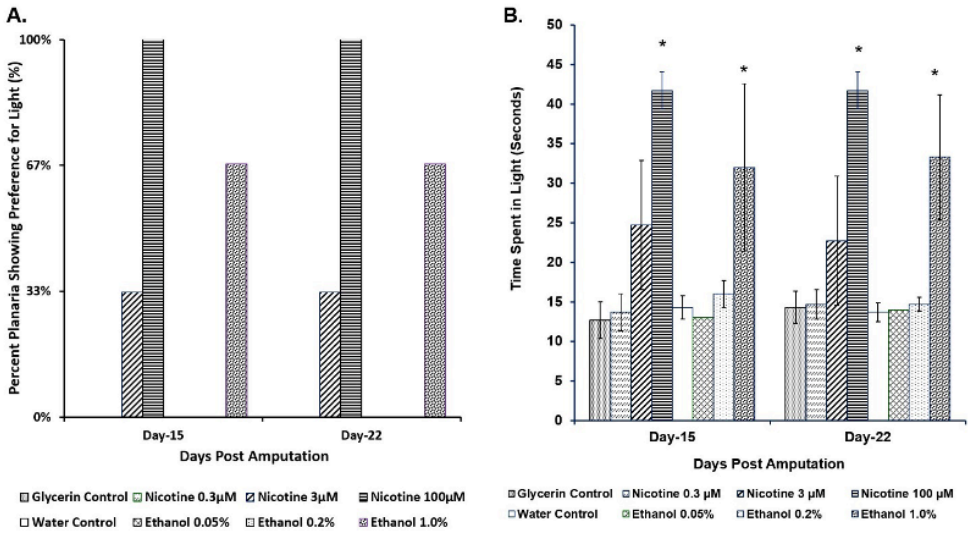


**Figure 6. Effect of parental exposure to nicotine or ethanol on light preference and time spent in light by regenerated planaria offspring.** (A) Bar graph depicting the effect of parental exposure to nicotine or ethanol on light preference by regenerated planaria offspring, showing the percent of planaria showing light preference on different days post-amputation. N=3 per group for all groups. (B) Bar graph showing the effect of parental exposure to nicotine or ethanol on time spent in light by regenerated planaria offspring, showing the mean number of seconds ± standard error spent in light on different days post-amputation. N=3 per group for all groups.

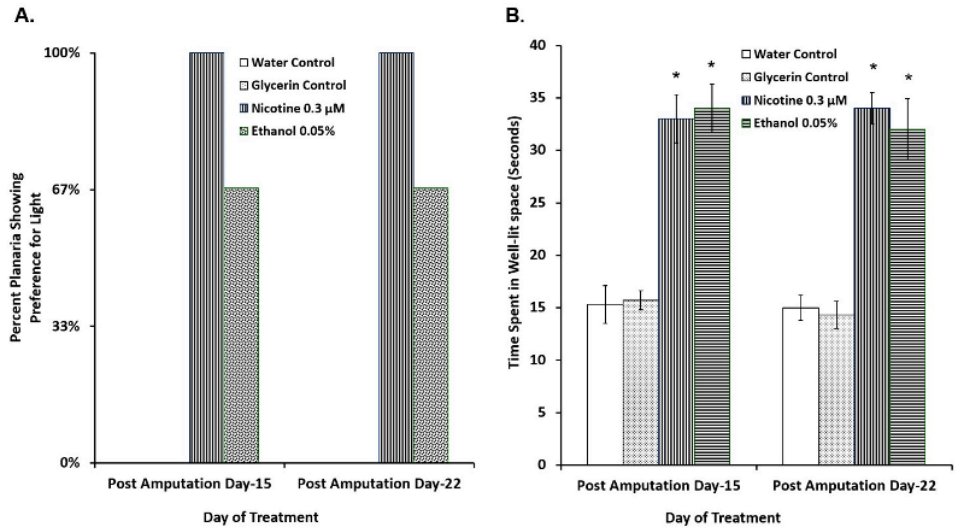


**Figure 7. Effect of direct nicotine or ethanol exposure during regeneration on light preference and time spent in light by regenerated planarian offspring.** (A) Bar graph depicting the effect of direct nicotine or ethanol exposure during regeneration on light preference by regenerated planarian offspring. N=3 per group for all groups. (B) Bar graph showing the effect of direct nicotine or ethanol exposure during regeneration on time spent in light by regenerated planarian offspring on time spent in light by regenerating planaria ± standard error. N=3 per group for all groups.

## DISCUSSION

This is the first study to demonstrate brown planaria as an innovative model to test chronic abuse liability in adults, and to test the transmission of substance abuse potential from parents to offspring. In this study, parent planaria exposed to high concentrations of nicotine (3 µM and 100 µM) or ethanol (0.2% and 1%) demonstrated C shape hyperkinesia, consistent with planarian response to substances of abuse including nicotine, ethanol, cocaine, glutamate and methamphetamine (15). Although planaria have not been used for chronic or intergenerational substance abuse liability assessment studies before, planaria displayed enhanced motility and stereotypical activity, abstinence-related withdrawal, and behavioral sensitization to cocaine; cross-sensitization to cocaine and glutamate; and CPP to methamphetamine in previous short-term studies similar to the observations in our study (15).

Development of tolerance is a key feature of substances of abuse, leading to progressive increases in intake. eventually leading to fatal overdosing (23). Tolerance to nicotine in planaria was observed at lower doses (3 µM) than what was observed previously (1-3 mM), likely due to the repeated nature and longer duration (10 days) of conditioning in our study (15). It is worth noting that there was no evidence of

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general toxicity at the concentrations of nicotine and ethanol used in our definitive experiment, including any impact on mobility and the regeneration, which seemed to happen at a similar pace between groups, with the presence of head and eyes in all the planaria by day 14.

We demonstrated that exposure of parent planaria to high concentration of nicotine (3 µM or 100 µM) resulted in CPP in both parent planaria and their regenerated offspring, even without direct exposure of the regenerated offspring to nicotine or ethanol during regeneration. This is consistent with the transmission of tobacco abuse behavior from human parents to offspring. Alcoholism in parents is known to increase the risk for alcohol dependence in children, however, it is unclear if this is due to genetic risk factors or environmental risk factors or both, as these studies in humans are confounded by environmental and socioeconomic factors (9). However, we could not find any reports of animal studies testing whether parental use of nicotine or alcohol predisposes offspring to nicotine dependence. Our study also demonstrated that the regenerating offspring exhibited CPP when exposed to a concentration of nicotine (0.3 µM) that was lower than the concentration that elicited C-shape hyperkinesia or CPP in parent planaria, indicating increased sensitivity to nicotine induced CPP in regenerating offspring of parent planaria exposed to nicotine. Remarkably, the concentration of nicotine that caused CPP in regenerated offspring was similar to the plasma concentrations of nicotine in smokers (21).

The study concluded that regenerated planaria offspring are more sensitive than parents and seem to transfer the sensitivity from parents to regenerated offspring, but it is outside the scope of the experiment to imply any genetic link for this phenomenon. Many recent studies have pointed out that epigenetic changes caused by chemicals in the germline could be playing an important role in the intergenerational transfer (24). Studies have shown that planaria also use similar epigenetic modifications to regulate transmission of memories across generations (25). While regenerated offspring are not generated via sexual reproduction, publications have demonstrated that regenerating offspring are not genetically identical to the parent planaria (25–27). It is also worth noting that there are clear differences in data between parent planaria and regenerated offspring in our study. For instance, parent planaria were not conditioned at the lowest dose tested (0.3 µM nicotine; 0.05% ethanol), but regenerated offspring were. Nicotine, caused conditioning in 100% parent planaria at 3 µM but only 33% of regenerated offspring showed light preference. Similarly, 0.2% ethanol caused conditioning in 100% parent planaria, but not in any of their regenerated offspring. If the results in regenerated offspring were just because they were same as parents, the observations would have been identical between parents and offspring.

In our study, parental exposure to nicotine or ethanol caused increased sensitivity to these substances in regenerating planaria offspring when they were exposed to these substances, showing that the regenerated offspring are predisposed with a memory from parental substance abuse. Interestingly, parental exposure to these substances caused conditioning in offspring even when regenerated planaria offspring were not exposed to nicotine or alcohol during its development. This suggests that parental substance abuse could predispose offspring, even when the parents are not abusing substance while the offspring is developing. Overall, our results demonstrate that intergenerational transfer of substance abuse potential of known addictive substances such as nicotine and ethanol occur in planaria. Considering the risk parental substance abuse poses for offspring, planaria can be used as an innovative model to test the transmission of substance abuse liability from parents to offspring in a faster and cost-effective manner than traditional animal testing. Further investigations to understand the molecular mechanism behind these observations in regenerating planaria will improve our understanding of the model and its relevance for human risk assessment.

## MATERIALS AND METHODS

### Preparation of test items

Nicotine, commercially available as a solution in vegetable glycerin (Central Vapors; 100 mg/mL or 6.16 x 10-1M) was further diluted in spring water (PICS brand by Price chopper) to achieve appropriate dilutions. In the definitive experiments, the nicotine concentrations used were 100, 3 and 0.3 µM. Similar to nicotine, anhydrous ethanol (Carolina biological supply company, catalog #861298), was diluted in spring water to achieve dilutions appropriate for experiments. In the definitive experiment, the ethanol concentrations used were 0.05%, 0.2% and 1%. Control groups included spring water (for ethanol groups) and dilutions of glycerin corresponding to the highest concentration of nicotine (for nicotine groups) used in each experiment. These test items at various concentrations were considered as the independent variables.

### Maintenance of planaria

Brown planaria (*Dugesia tigrine)* were procured from Carolina Biologics and maintained according to their instructions. Planaria were kept in commercially available spring water in clean glass or plastic containers with a loose-fitting lid for air flow. Planaria were maintained on a weekly diet of hard-boiled egg yolks. Planaria were fed 2 days before the starting the definitive experiments. The parent planaria were fed after light conditioning for 10 days, during the 24 hours prior to amputation in the definitive experiment.

### Exposing planaria to test items

Parent planaria were exposed to varying concentrations of nicotine or ethanol and the respective vehicle controls in 6-well plates (Carolina Biological Supply Company). The final volume of test items or vehicle controls in each well was 3 mL. In the definitive experiment to identify the intergenerational transmission of the effects of nicotine or ethanol in planaria, an n = 6 of planaria was used for the vehicle controls and the lowest concentration of the test items, and an n = 3 of planaria were used for the other concentrations of the test items (**Figure 1**).

### Light conditioning of planaria

A light box for conditioning planaria was made by inserting 4 light-emitting diode (LED) bulbs (Ecosmart), each emitting 840 lumens on the inside of a cardboard box. Six-well plates containing test items and planaria to be conditioned were placed on a transparent glass sheet, positioned on top of the box. The plates were covered on the top using a similar light emitting box (**Figure 8**). For light conditioning, parent planaria were exposed to either nicotine, ethanol, or their respective

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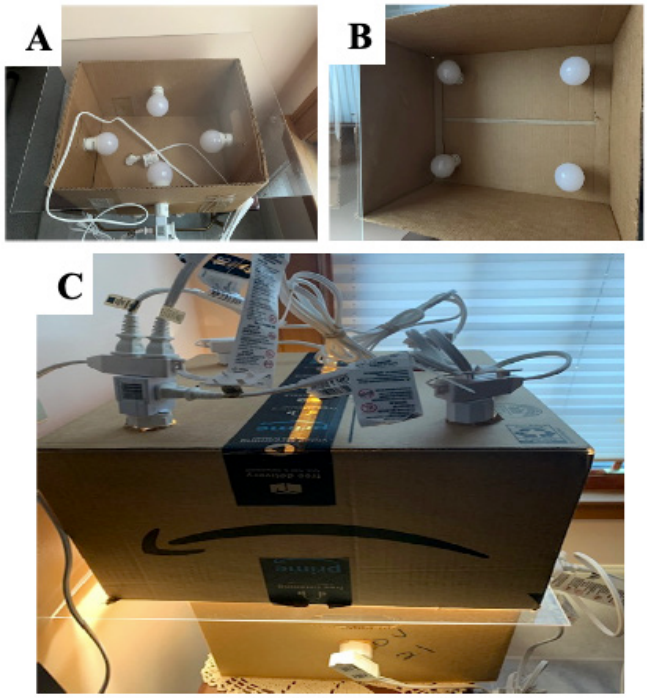


**Figure 8. Pictures of light box used for conditioned place preference of planaria.** Light box for conditioning planaria was made using cardboard box as pictured above. A – Bottom portion of the light box with transparent glass for placing planaria containing 6-well plates or petri dishes. B – Top portion of the light box. C – Light box in use for conditioned place preference of planaria.

vehicle controls in the presence of light for an hour daily. After exposing to light, the 6-well plates were moved from the light box and the test items were removed from the wells. Then, the planaria were washed 3 times and kept in spring water in a dark box for 22 hours. This was repeated for 10 days. A subset of regenerating planaria were conditioned similarly for 14 days.

### Assessment of C-shape hyperkinesia (C-curling)

The acute planarian response to nicotine or ethanol exposure was evaluated by daily measurement of the C-shape hyperkinesia (number of times planaria curled into a "C") in parent planaria within 90 seconds of being exposed to test items or their respective vehicle controls.

### Assessment of conditioned place preference (CPP)

Conditioned place preference (CPP) of planaria towards a well-lit environment was evaluated by measuring the following parameters (dependent variables): light preference and time spent in light. During phase 1, light preference and time spent in light was evaluated daily in parent (adult) planaria during the 10-days of light conditioning, ~22 hours after light conditioning (**Figure 1**). The light preference and time spent in light for regenerated offspring, were evaluated on day 15 and day 22 post amputation during phase 2 (**Figure 5**). Notably, there is no exposure to nicotine or ethanol during the time when light preference and time spent in light are tested in parents or regenerated offspring. To evaluate light preference and time spent in light, a polystyrene square integrid petri dish (Carolina Biological Supply Company catalog # 741470) containing spring water was inserted up to its middle into a small box to create a well-lit and dark space. Planarian was introduced at dark end of the petri dish to assess light preference and at the intersection of dark and well-lit regions to evaluate time spent in light. Petri dish was observed for 90 seconds to assess light preference, followed by 90 seconds to assess time spent in light, on the light box with the bottom lights turned on. The observation of light preference was made if the entire planaria moved into the well-lit region of the petri dish and the time planaria spent in the well-lit region of the petri dish was measured (in seconds) using a stopwatch (**Figure 1**).

### Amputation of parent planaria and generation of regenerated offspring

After light conditioning of parent planaria for 10 days, the head of these planaria were amputated and regenerated offspring were generated by allowing the tail to regenerate and form a new head. For amputation, individual planaria were transferred from the container using a clean paint brush (Crafter's Square, Greenbrier International) and placed on a glass histology slide (Carolina Biological Supply Company, Catalog # 632010) wrapped in wax paper (Greenbrier International). Then, a drop of spring water was placed on the planaria, allowing it to stretch, which allowed quick amputation of the head. Once stretched, the head of planaria was amputated using a scalpel. Tails from parent planaria (n = 3 per group) conditioned to light in the presence of vehicle controls and three different concentrations each of nicotine or ethanol were maintained in spring water and kept in a dark box for 14 days. Tails from a subset of parent planaria (n = 3 per group) conditioned to light in the presence of vehicle controls and lowest concentration of nicotine (0.3 µM) or ethanol (0.05%), were allowed to regenerate into offspring with continued light conditioning for 1 hour daily, similar to parent planaria, in the presence of low concentrations of nicotine or ethanol for 14 days (**Figure 5**). The viability and growth of the regenerating tails were confirmed by once daily examination, under a Universal Serial Bus (USB) digital microscope, without removing them from the wells.

### Statistical analysis

Mean frequency and standard deviation for each day were calculated for C-shape hyperkinesia and time spent in light in parent planaria during the 10 days of light conditioning. Similar analysis was performed for the regenerated offspring on day 15 and day 22 post amputation. Light preference of planaria (during 10 days of light conditioning for the parent planaria and on day 15 and day 22 post amputation for the regenerated offspring) were expressed as a percentage of the number of planaria preferring the well-lit region, against the total number of planaria in the group. Statistical significance of the difference in time spent in light between test item treated groups and control groups was evaluated in the definitive experiment using the t-test function [T.TEST(*array1,array2,Tails,type*)] in Microsoft Excel. Since the values for the water control and glycerin control groups across all time points (144 observations) were similar, both spring water and glycerin groups together was considered as *Array1. Array2* was each of the test item treated group. The value for "Tails" was 2 since a 2-tail test was conducted, and the value for

https://doi.org/10.59720/23-014

"type" was 2 meaning the variance between samples were similar. A p-value of < 0.001 meant that the probability of observations in the nicotine or ethanol groups occurring by random chance was less than 1 in 1000, indicating that the difference between the compared groups was statistically significant.

**Received:** January 17, 2023
**Accepted:** September 24, 2023
**Published:** July 02, 2024

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