Reinforcing and anxiolytic-like effects of alcohol in planaria require μ-opioid receptor activation

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Statement of Sources

I declare that this report is my own original work and that contributions by others have been duly acknowledged

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Abstract

Planaria have been used for decades as subjects for psychopharmacology research, predominantly in addiction studies involving dopamine agonists. However, this research largely focused on classical conditioning, with comparatively few studies examining how planaria behaviour is altered by alcohol administration. In humans and other mammals, ethanol enhances dopamine transmission via endogenous opioid release. This study sought to investigate the link between ethanol and endogenous opioids in planaria in a novel operant conditioning paradigm and avoidance task. Study 1 was used to determine whether 1% or 2% ethanol concentrations induced significant conditioning, with 2% showing greater effectiveness. Study 2 investigated operant conditioning with ethanol (2%), finding significant results after 4 days of reinforcement. Study 3 investigated whether the mu-opioid receptor antagonist naloxone affected conditioning induced by ethanol. Results indicated that naloxone blocked the acquisition of discrimination learning with 2% ethanol, suggesting that alcohol reinforcement in planaria necessitates mu-opioid receptor activation. Study 4 investigated the anxiolytic effect of ethanol in planaria, finding significant increases in light tolerance when ethanol was administered. These novel results indicate that planaria are a valuable animal model for ethanol research and that potential future alcohol abuse medications might be validly tested in this species for preliminary efficacy studies.

Keywords: naloxone, operant conditioning, psychopharmacology, addiction, anxiolytic

While not as common as rodents or other animals in psychopharmacological studies, the planarian family of flatworms is a useful, cost-effective, and methodologically simple model system with which to study reward mechanisms, including drugs of abuse (Raffa & Rawls, 2008). The usefulness of planaria for the study of addiction is due in part to their behavioural and neurological simplicity but also the similarities with animals possessing more developed nervous systems (Barron et al, 2010; Raffa & Rawls, 2008). An added benefit of using planaria in psychopharmacology research is that invertebrates do not require ethics approval since they are regarded as a non-sentient species (Raffa & Rawls, 2008).

The nervous system of planaria is bilateral, with a high concentration of ganglia found in the head and a differentiation between dorsal and ventral areas. Although basic, planaria possess eyes which are capable of distinguishing light and dark, as well as receptors for tactile sense and chemoreceptors (Sarnat & Netsky, 1985). Also present in planaria are many of the same pharmacological receptors found in humans and other mammals, including serotonin (5-hydroxytryptamine or 5HT) dopamine, GABA, glutamate, cholinergic and opioid receptors (Buttarelli et al, 2008). Full-grown planaria are typically 10-15mm long and can be maintained with weekly feeding of boiled egg yolk or other protein-rich foods. This amount of feeding is sufficient for sustaining over 100 planaria at a time, in contrast to adult rodents of much greater size which require significantly more space and resources to keep alive and healthy. Despite their small size, planaria have a high brain-bodyweight ratio, with newborn planaria head and brain sizes proportionally larger than adults, in a similar manner to human infants and adults (Sarnat & Netsky, 1985). The bi-hemispheric neuronal masses of the planaria head narrow as they continue down the length of the body, effectively forming a sort of spine. Coupled with their prototypical nervous systems, planaria are also well known for their self-restorative capabilities, able to recover after being totally severed through the sagittal or transverse planes and regenerating into two complete worms (Reddien, 2018).

Both classical (Amaning-Kwarteng et al, 2017; Hutchinson et al, 2015; Mohammad Jawad et al, 2018; Raffa et al, 2013; Raffa & Valdez, 2001; Rawls et al, 2011, Tallarida et al, 2014) and operant conditioning have been demonstrated in planaria, although only one operant study, involving avoidance behaviour, appears to have been published (Lee, 1963). This study also received strong criticism later in the same year due to potential methodological flaws (Halas, 1963). It is worth noting that the operant conditioning (Lee, 1963) used a negative reinforcement paradigm, wherein an aversive stimulus was removed, while no studies to date appear to have been conducted with positive reinforcement – that is, exposure to a rewarding stimulus after a behaviour.

Classical conditioning has been established in planaria with natural reinforcers like sucrose (Mohammad Jawad, 2019; Mohammad Jawad et al, 2018) as well as drugs of abuse including nicotine (Rawls et al, 2011), cocaine (Amaning-Kwarteng et al, 2017; Tallarida et al, 2014), ethanol (Tallarida et al, 2014) and amphetamine (Raffa et al, 2013). Some of the behavioural changes exhibited by planaria in the presence of drugs include physiological effects of withdrawal. Examples include measuring hypo-locomotion with the opiate mephedrone (Hutchinson et al, 2015) and cocaine (Raffa & Valdez, 2001).

Conditioned place preference is a well-established paradigm in psychopharmacological research to study reward mechanisms. In a study assessing cocaine conditioned place-preference (Amaning-Kwarteng et al, 2017), planaria were placed in petri dishes with two distinctly textured halves – a rough and smooth side. Planaria were exposed daily in separate sessions to the naturally preferred side in the presence of water only, and to the non-preferred side in the presence of cocaine. Following conditioning, planaria demonstrated a robust conditioned place preference. These researchers also showed that cocaine place preference could be reinstated after extinction with both cocaine and methamphetamine. These experiments demonstrated the acquisition, extinction and

reinstatement of implicit long-term associative memory for environmental cues related to drug exposure. Because of the parallels between planaria and mammals in terms of neurochemical mechanisms and associative processes, the low costs and ease with which they can be studied in large numbers, planaria may therefore constitute an effective model to investigate drug-related behavioural and neurochemical processes.

Although animal models of operant self-administration are the "gold standard" in drug research (Haney & Spealman, 2008), there are surprisingly few studies which have demonstrated this with invertebrates, and none so far with planaria specifically. While no studies have yet been published using reinforcing agents to produce an operant conditioning response in planaria, crayfish have been shown to self-administer amphetamine (Datta et al, 2018) and starved ants given the choice between the natural reinforcer of sucrose and the opiate morphine chose to self-administer the drug, despite a lack of caloric value (Entler et al, 2016).

Ethanol is one of the most widely consumed recreational drugs across the world and consequently, one of the most widely abused. Data from two surveys and amounting to over 79,000 participants show that overall use, high-risk drinking and alcohol use disorder have been increasing in the United States since the early 21st century (Grant et al, 2017). Scheideler and Klein (2018) have shown that although alcohol is classified as a class 1 carcinogen, indicating that it belongs to a group of substances carrying the highest level of risk for cancer development, knowledge regarding this risk factor of alcohol consumption is relatively low worldwide, though moderately higher in Australia, Morocco and the United Kingdom. Livingston et al (2018) reported that alcohol consumption in Australia has trended down since 2007-2008, due primarily to lower consumption in younger people. Despite this national trend, Axley et al (2019) found that alcohol consumption worldwide increased by

25% from 1990-2016 and was the seventh highest risk factor for disability-adjusted life expectancy.

Research in Australia has found that even when using conservative methods to estimate the negative societal effects of alcohol consumption, tax revenue from the sale of alcohol was significantly lower than the costs (Manning et al, 2013). These included costs in the health and justice systems, road accidents involving alcohol and productivity losses. An international meta-analysis of 29 studies (Manthey et al, 2019) estimated that mean costs of alcohol consumption were equivalent to 1.5% of GDP, primarily in lost productivity, but almost 40% of the costs were determined to be direct. A meta-analysis of European Union costs comparing illicit drugs to tobacco and alcohol (Barrio et al, 2017) found that alcohol had the highest cost per capita in 2014, although the authors stressed that standardised methodologies were needed to improve comparisons. A meta-analysis of studies from the United States, Canada, Sweden and New Zealand on the impact of foetal alcohol spectrum disorder (Greenmayer et al, 2018) found that the mean annual cost per child was $22,810 and $24,308 per adult. Collectively, these studies and others indicate that ethanol constitutes a major health burden on society.

While ethanol is a strong modulator of of GABA receptors, the drug has secondary effects on both opioid and dopaminergic activity, primarily due to disinhibition of these systems, with ethanol addiction in humans seemingly dependent upon the GABA- opioid-dopamine chain (Cowen & Lawrence, 1999; Peterson et al, 1996). GABA (gamma-aminobutyric acid) is the primary inhibitory neurotransmitter in humans (Ngo & Vo, 2019). It is also known to be one of the first neurotransmitters present in developing nervous systems (Wang & Kriegstein, 2009). Research by Stagg et al (2011) suggests that due to its importance in motor cortex plasticity, individual sensitivity to modification of the GABA system influences motor learning in healthy adults. A recent study by Dolfen et al (2021)

showed that stress modulated the relationship between striatal GABA and the plasticity of both the striatum and hippocampus when learning a motor sequence. Further research into clinical conditions, with cognitive deficits as a primary symptom, has also suggested a significant role for GABA-induced disinhibition in the treatment of these conditions (Möhler & Rudolph, 2017). A review of the relevant animal and human literature (Kalueff & Nutt, 1996) indicated that GABA is a major factor in the relationship between anxiety and memory. Despite the highly specialised role played by GABA in memory formation, this neurotransmitter has been shown to be involved in functions as broad as autoimmune inflammation (Bhat et al, 2010), the perception of pain (Enna & McCarson, 2006) and multiple effects on neurogenesis in embryonic nervous systems (Wang & Kriegstein, 2009).

Endogenous opioids are involved in important functions including nociception (Budai & Fields, 1998; Roques et al, 2012; Stein et al, 1990), the immune and neuroendocrine systems (Carr, 1991; Stein et al, 1990), stress response (Drolet et al, 2001), hormone release (Miller, 1980), cardiovascular and respiratory function, thermoregulation, memory and sexual, locomotor and aggressive behaviour (Khachaturian et al, 1993). More recently, their role in mood disorders has been emphasized, with some authors suggesting that novel antidepressants acting on opioid receptors could be employed (Lutz & Kieffer, 2013).

Dopamine is the principal neurotransmitter responsible for reward, motivation, and appetite satiation, and is related closely to the limbic system which regulates basic homeostatic functions like food and water consumption, sleep and reproduction (Barron et al, 2010). Stimulant compounds such as amphetamine and modafinil induce their effect through increasing dopamine (Wisor et al, 2001). Addictive compounds and behaviours rely on learned responses to stimuli produced by long-term potentiation (LTP), an effect which has been shown to be contingent on dopamine and glutamate (Wolf et al, 2004). The mesocortical and mesolimbic pathways innervating the mammalian forebrain have been consistently

associated with addictive behaviour. Dopamine in these neural pathways is directly involved in executive function, cognition and emotional regulation (Mylecharane, 1996; Ranaldi, 2014).

In humans, evidence suggests that GABA directly affects the nucleus accumbens (NAcc) and ventral tegmental area (VTA), which are both intrinsic parts of the reward-related mesocorticolimbic dopamine system (Xi & Stein, 2002). Gianoulakis (1996) found that individuals at high risk of developing alcoholism based on family history, had a lower basal level of beta-endorphins in blood plasma, but greater release after consuming ethanol. Evidence for ethanol increasing the activity of mu- and delta-opioid receptors in both the VTA and NAcc via disinhibition was also noted in rat and mice (Froelich et al, 1987). There is also evidence (Cowen & Lawrence, 1999) that dopaminergic pathways 'downstream' of the mesolimbic system are affected by ethanol intake and assist forming memory and shaping attention around stimuli related to ethanol. For those at high risk of developing alcoholism due to a family history of abuse, one warning sign is that alcohol and the associated increase in endogenous opioids may act as a psychomotor stimulant, likely due to the subsequent dopamine release, causing an increase in heart rate (Peterson et al, 1996; Pihl & Peterson, 1995). Research on rats offers support to the theory that endogenous opioids are an integral part to self-administration in mammals. Co-administration of the mu-opioid agonist morphine with alcohol has been found to increase ethanol consumption in an operant paradigm, while co-administration of the mu-opioid antagonist naloxone has been shown to result in daily reductions in ethanol self-administration (Hubbell et al, 1986).

Consumption of ethanol also initiates a potent anxiolytic effect in humans (Knight et al, 2020). Research has also demonstrated a similar effect of GABA agonists on planaria negative phototaxis – an instinctual preference for darker environments when the choice is available – showing that both ethanol and diazepam, a clinically utilised benzodiazepine,

increased time spent in the light (Zewde et al, 2018). Evidence from studies with humans suggests that self-medication with alcohol for its anxiolytic effects is a common motivation for use (Chutuape & de Wit, 1995). While most drugs prescribed presently and historically as anxiolytics such as benzodiazepines and barbiturates exert their function on GABA, with a well-established relationship of this transmitter to stress and anxiety (Kalueff & Nutt, 1996), the role of opioid receptor activation has also been strongly implicated in mediating this effect (Colasanti et al, 2011; Drolet et al, 2001). If GABA agonists reliably demonstrate anxiolytic effects, this may be at least partly due to the function of opioids, rather than exclusively GABAergic compounds, due to the cascade effect of GABA-reliant disinhibition often resulting in amplified opioid release (Xi & Stein, 2002).

Findings in animal models offer support for well-established theories relating to the difficulty of overcoming a drug addiction being at least partly related to environmental triggers that are implicitly linked to drug use and act as primers for this behaviour (Stacy & Wiers, 2006). Stress and anxiety are two factors which have been associated with GABA agonist self-administration in both humans (Chutuape & de Wit, 1995; Piazza & Moal, 1998; Turner et al, 2018) and in rodent studies (Wilson et al, 2004). A study of the anxiolytic and stimulant effects of ethanol and benzodiazepines in mice found that the anxiolytic effect of both substances diminished over time, while the stimulant effect remained (Boerngen-Lacerda & Souza-Formigoni, 2000). Mood disorders may also potentially influence self-medication due to the euphoric effects of alcohol in humans Turner et al, 2018). It should be noted however that stress, anxiety and mood disorders do not inevitably lead to self-medication (Levy, 2019). An issue has also been raised concerning pharmacotherapeutic treatments for substance use disorder being developed in animal models, while subsequently not translating to human research and clinical applications with the expected efficacy (Haney & Spealman, 2008).

It is worth noting that drug abuse and addiction is influenced by a number of factors, including social, cultural, economic, individual differences and neurological sensitivity, among others (Merikangas & McClair, 2012; Wills et al, 1996). Epigenetic interactions between these factors play an important role as well (Cadet et al, 2016), making the problem more difficult to understand and solve. Especially noteworthy, social isolation has been studied in rodent models and has a strong influence on drug seeking behaviour for opiate users (Christie, 2021; Raz & Berger, 2010) and nicotine (Hofford, 2021), amongst others. A general increased sensitivity to dopamine, and consequently stimulants, has been shown to result from developmental social isolation (Yorgason et al, 2016).

A commonly used pharmacological intervention for reducing or eliminating alcohol consumption in humans is naloxone (Froelich et al, 1987), which is an antagonist of opioid, primarily mu, receptors. Naloxone is also used to facilitate both cessation of opiate abuse and reverse the effects of overdoses (Krieter et al, 2016). Because of the antagonistic action of naloxone on opioid receptors, it appears to reduce alcohol consumption by blocking the effects of ethanol on opioidergic and dopaminergic pathways mediating reinforcement (Froehlich et al, 1991). A side effect of naloxone is increased sensitivity to pain, which is likely due to its action of blocking endogenous opioids. For this reason, experimental attempts have been made to use naloxone in the treatment of congenital insensitivity to pain, although without meaningful behavioural change in patients with this specific condition (Protheroe, 1991). Because of the well-established clinical history and known pharmacological properties of naloxone as an opioid antagonist, primarily of mu-opioid receptors, this compound was chosen to test our hypotheses in the present study.

With this research, I sought to establish new behavioural paradigms to explore the similarities between the mechanisms of action of ethanol in planaria with those of humans and other mammals. First, the reinforcing effect of ethanol was studied in a native species of

planaria, which has never been utilised in drug studies. This was achieved using a novel operant conditioning paradigm based on spatial discrimination. Secondly, naloxone was used to examine the possible involvement of mu-opioid receptors in ethanol reinforcement. Control groups and contrast groups with water and the natural reinforcer sucrose were also included for comparison with the ethanol groups. A final study sought to ascertain whether ethanol induces an anxiolytic-like response in planaria similar to humans and whether the effect of ethanol modulated by mu-opioid receptors.

Our hypotheses were logical extensions of evidence found by previous studies which sought to uncover the means by which ethanol elicits reinforcing effects in mammalian species. Because the same neurotransmitter systems may be responsible for drug-induced conditioning in planaria as in higher order animals – specifically opioids and dopamine – we hypothesised that administering alcohol in a planaria's non-preferred environment would result in an operantly conditioned response (study 1 and 2) for that environment and that naloxone would interfere with the acquisition of this operant response (study 3). The operationalised parameters of operant behaviour were the daily choice rate of the drug-associated arm, measured first as a baseline to determine natural preference, and subsequently during conditioning to measure the positive association and hypothesised change in choice.

Planaria were expected to show a stronger response to 2% ethanol in study 1, a stronger conditioning response for ethanol than sucrose in study 2 and no significant change in drug-paired arm choice was hypothesised for the water only and ethanol/naloxone groups in study 3. Study 4 was expected to show a significantly higher proportion of time spent in the light for the ethanol group compared to the water and ethanol/naloxone only group, as we hypothesised that naloxone would interfere with the anxiolytic-like effects of ethanol, due to evidence suggesting that opioids play a crucial role in mediating this effect.

Method

Subjects

A total of 78 planaria were utilised across all experiments. The planaria used were acquired from Southern Biological (Victoria, Australia). Upon arrival at the laboratory, planaria were kept in groups of 10 in plastic vials and collectively fed weekly with boiled egg yolk before being returned to the vials in groups of 10. Planaria were kept at all times in the Invertebrate Psychopharmacology Laboratory of the Psychology Research Centre, with room and water temperature maintained between 21-23ºC.

Materials

Ethanol used in this study was 100% undenatured, acquired from Chem-Supply (AU). Sucrose and naloxone (naloxone hydrochloride dehydrate) were purchased from Sigma-Aldrich (AU). The concentrations tested were 1% and 2% for alcohol, 20µM naloxone and 2% sucrose.

Self-administration studies utilised Y-mazes with a pluggable intersection (see Figure 1) for all conditions. The first chamber of the maze was 25mm in length, the intersection 7mm diameter and each arm 25mm in length. The plastic plates from which the mazes were made were 80mm squares. 1% and 2% concentrations of ethanol were administered in study 1, 2% ethanol and 2% sucrose in study 2, naloxone in a 20µM/2ml spring water mix was used to fill mazes in study 3 for the 2% ethanol group, while spring water was used in the water only control group.

Figure 1: A Y-maze used in experiments 1-3.

The light-dark study of anxiety-like behaviour required a lightbox (see Figure 2) and three petri dishes, filled 8ml of liquid. Liquid was either water in the water only group, water with a 2% concentration of ethanol in the ethanol group and 2% ethanol with 20µM naloxone for the naloxone group.

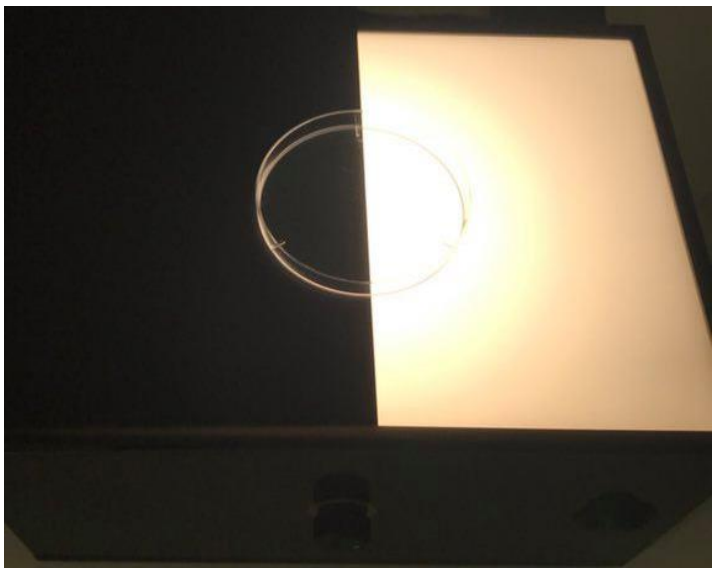

Figure 2: The lightbox used in study 4.

In all studies, fine art brushes were used to transport planaria from the multi-well plates into the test areas and back again. Uncontaminated spring water in a petri dish was

used to rinse planaria and the brushes between trials. Studies using the Y-mazes also required paper towel to thoroughly dry the mazes between trials and avoid cross-test contamination.

Procedure

The first set of experiments were designed to investigate the reinforcing effect of ethanol in planaria. In experiments 1-3, planaria were placed into a Y-maze for each discrimination learning trial. Each maze was filled with 2ml of spring water. A white LED built into the dish at the base of the Y provided a stimulus from which planaria would typically swim away – due to negative phototaxis – into one of the arms of the Y. At this point a plug was inserted to close the intersection of the maze. Experiments were conducted in a dimly lit room to avoid light pollution during trials affecting planaria sensitivity and arm choice.

When the intersection of a Y-maze was blocked, each arm held 750µl of water, so a mixture of 50µl of water and ethanol was administered during trials into the DP compartment. The 50µl mix contained a proportion of ethanol such that the final 800µl volume contained either 1% or 2% alcohol – accordingly ethanol-water concentrations were 8:42µl for 1% and 16:34µl for 2% tests. In study 1, we conducted a dose-response to determine which concentration of alcohol – 1% or 2% – would be utilized in the following studies. Sucrose, a natural reinforcer, was used at a 2% concentration was to compare with ethanol. All solutions were administered at room temperature.

In study 3 we studied the role of mu-opioid receptors in the acquisition of operant conditioning mediated by ethanol. To this effect, a final concentration of 20µM of the mu antagonist naloxone was present in the 2ml of maze water during trials. This dose of naloxone has been previously found to disrupt morphine conditioning in planaria (unpublished observations, Canales lab, University of Leicester). The naloxone solution was

pre-mixed with spring water to reach the desired concentration and kept frozen in 50ml falcon tubes until the day of experiments. All spring water and water/naloxone solutions were administered at room temperature throughout the studies. A large jug was used to empty mazes and paper towel was used to dry them, for the purpose of ensuring no cross-contamination by residue between trials.

Methodologically, studies 1-3 were operant conditioning paradigms utilising a fixed ratio positive reinforcement schedule (reinforcement received for 100% of choices) with ethanol as the reinforcing agent.

In the second set of experiments (study 4) we studied the anxiolytic-like effects of ethanol on planaria using a light-dark paradigm. Over half of a lightbox, black paper thick enough to block the light was placed, with three petri dishes each positioned on the box so that half the dish received light and the other half was darkened. A total of 8ml of liquid was used in each petri dish, containing one of the three different treatments: 2% ethanol concentration, spring water only and spring water/naloxone with 2% ethanol. The ethanol solutions were mixed fresh on the day of study 4.

After selection and between experiments, planaria were housed individually in sterile multi-well dishes designed for cell culture. Planaria in study 1 were housed in a single plate with 24 wells, while subsequent studies housed planaria in plates with 6 large wells each. Planaria were assigned identification numbers once housed in the multi-well plates.

In study 1, 2 and 3, baseline choices for each arm were recorded across six trials conducted in two sessions for every planaria. Planaria showing no preference were randomly assigned to either the left or right arm, assigning equal numbers within an experimental group to each drug-paired (DP) arm. The non-preferred arm at baseline was the DP arm in all subsequent trials. Beginning at the same time of each day following baseline, planaria were

sequentially placed in the maze for three trials. All planaria completed their first trial before restarting at the beginning of the list, repeating this order for trials 2 and 3. After entering an arm in each trial, the plug was inserted to block the maze intersection and the light was switched off. Alcohol of 1% (study 1) and 2% concentrations (study 1, 2 and 3) and sucrose in a 2% solution (study 2) was immediately administered with a pipette if planaria chose the DP arm and no drug (vehicle) in the preferred arm. Planaria were kept in their chosen arm for 3 minutes after administration. After drug administration and planaria removal from the mazes, the mazes were emptied and thoroughly dried with paper towel before refilling with 2ml spring water (study 1, 2 and 3) or 2ml spring water/naloxone solution where required (study 3).

Study 4: Planaria were divided into three groups – water only, ethanol 2% only and ethanol 2% with 20μM/2ml naloxone/water – and placed along the dark/light half border of the corresponding dish, then left for 3 min trials. Time spent in the light (hypothetically aversive) side of the box was recorded. Each planaria completed 3 trials. A simple ANOVA was conducted, with total time spent in the light across the three trials as the independent and treatment group as the dependent variable.

Analysis

Statistical analysis was performed using the open-source statistical software Jamovi ver. 1.6.15, with the optional GAMLj package installed. Although it was initially planned to use a simple ANOVA, a general linear model was used to analyse results, due to this method being more robust against missing data caused by planaria not completing experiments. Results for planaria which never chose the DP arm in experimental trials were not included in final analyses. A simple ANOVA was used in study 4. All post-hoc tests were Bonferroni-corrected.

Experiment 1 results – Ethanol dose-response

Due to the spontaneous deaths of a significant number of planaria in this study, recording of data was stopped on day 3. Results of the general linear analysis showed that while the model was not significant ($F[8] = 1.87$, $p = 0.073$, $\eta^2p = 0.129$) and neither 1% nor 2% concentrations yet showed a significant increase in preference over baseline there was a consistently higher DP choice average for those in the 2% concentration (see Figure 3). Thus 2% was selected as the ethanol concentration for subsequent studies. The lack of significance was concluded to be due to the death of 10/12 planaria in the 2% group, and 9 deaths among the 1% group by day 4 of conditioning, resulting in a loss of statistical power.

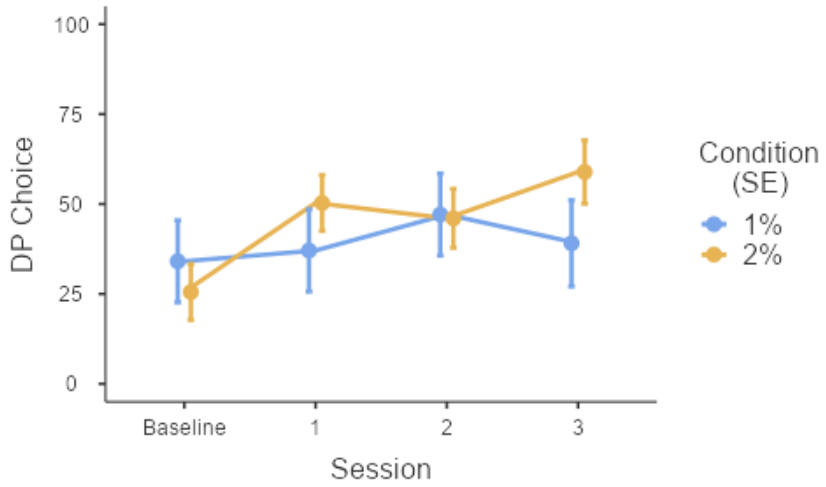

Figure 3: Pictured are the daily mean choice rates for the dose-response study. Although neither group reached a statistically significant difference from baseline, the 2% group had a more obvious and reliable tendency in DP choice (bars show SE).

The deaths were likely due to water contamination compounded by the use of small storage wells and lack of rinsing both planaria and the brush after removal from the maze. Subsequently, larger wells with 8ml of water were used for storing planaria and future studies included a rinsing stage of planaria in uncontaminated spring water between removal from the maze and their return to the multi-well plates.

Experiment 2 results – Ethanol/sucrose conditioning

Study 2 consisted of 24 planaria, which underwent the same baseline preference measurement for one arm or the other as those in the pilot study. The groups consisted of 12 planaria in the 2% alcohol group and 12 planaria in the 2% sucrose group. Due to spontaneous deaths of 4 planaria in the sucrose group before conditioning began and 2 more planaria failing to choose the DP arm and being discounted from the analysis, only 6 planaria remained for valid data. This was determined to be too few for a valuable analysis and the sucrose group was accordingly not included in subsequent analyses.

Conditioning followed the same protocol as study 1 for 4 days, when choice for the DP arm in planaria receiving ethanol reached a nearly 80% rate. Results of planaria in the 2% group from the initial study were included in the analysis for added power.

Overall the general linear model analysis was significant ($F[5] = 3.63$, $p = 0.005$, $\eta^2p = 0.183$). Bonferroni-adjusted post hoc tests found significance between the baseline and day 4 DP choice averages ($t[81] = –3.98$, $p = 0.002$). The average choice rate of the group on this day was 79.4% (95% CI [57.70, 100*], $SE = 10.93$) while baseline average was only 26.6% (95% CI [11.60, 41.60], $SE = 7.53$), amounting to an increase of 52.8% in choice rate for the 2% ethanol group. These results show that 2% ethanol effectively produced an operant conditioning response in planaria (Figure 4). (*value determined by jamovi was above 100%)

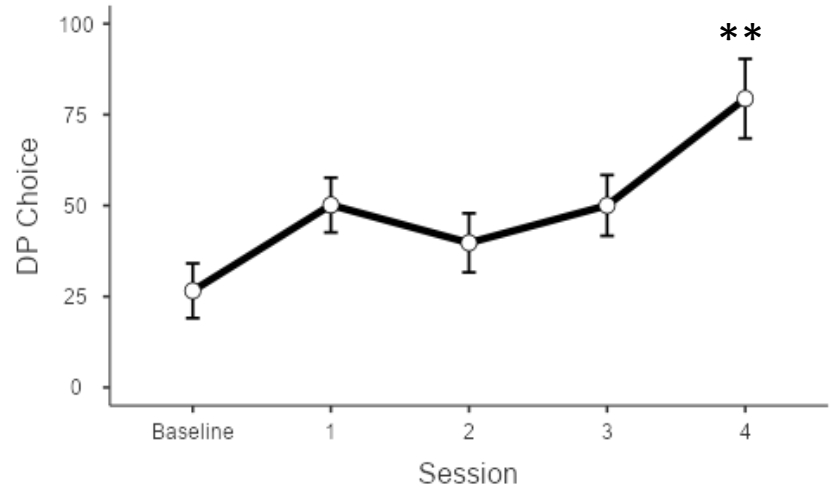

Figure 4: DP choice rate for planaria in the 2% ethanol condition of study 1 and 2 – data from two baseline (combined marker) and four conditioning sessions (bars show SE). Stars to highlight significant difference (p = 0.002).

Experiment 3 results – Effect of naloxone on conditioning

A control group (n = 12) receiving water only was included to provide a comparison for the naloxone group (n = 18), with these groups not expected to differ significantly (i.e., both results to show no significant change in preference for the DP arm over baseline). The study involved 30 planaria, with 16 planaria surviving to provide data beyond baseline in the ethanol/naloxone group and 11 in the water only group.

In this experiment, planaria in the water only and ethanol/naloxone groups did not show the ethanol-induced operant conditioning effect that had been observed in study 2 when ethanol was administered alone in the DP arm (Figure 5). This was evidenced by a lack of significant difference between baseline averages (M = 27.3, 95% CI [13.29, 41.30], SE = 7.06) and day 4 of conditioning (M = 34.1, (95% CI [19.41, 48.80] SE = 7.41) with a Bonferroni adjustment to the alpha (t[120] = –0.71, p = 1.00). Planaria in the water control group also demonstrated no significant increase in choice rate for the DP arm (t[120] = 2.26, p = 1.00) between baseline (M = 29.9, (95% CI [9.50, 50.20], SE = 10.27) and session 4 (M =

56.9, 95% CI [36.58, 77.30], *SE* = 10.27). The combined results of experiments 2 and 3 are shown in Figure 6.

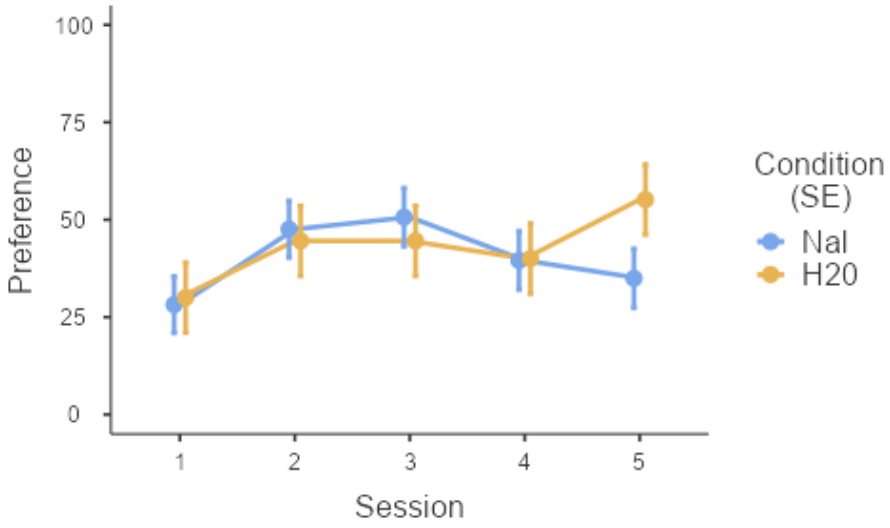

Figure 5: The trends for naloxone and water only groups, demonstrating no significant increase in DP choice rate across the sessions (bars show SE).

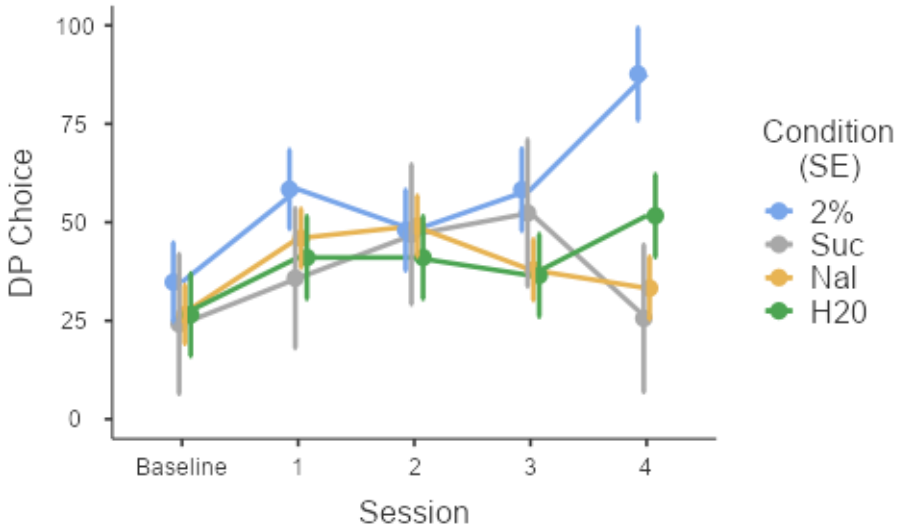

Figure 6: All four-day experimental group results (bars show SE).

Experiment 4 results – Anxiolytic-like response to ethanol

This experiment tested the anxiolytic-like effect of ethanol in planaria in a light-dark paradigm, as well as investigating its potential regulation by mu-opioid receptor activation.

The study consisted of three groups, with 9 planaria in the water only, 10 in the ethanol/naloxone group and 8 in the ethanol group. When initially analysing the results, there were two extreme outliers (outside 99.9% CIs) in the ethanol group and one in the water only group. The water outlier was above the group mean, while there was an outlier in both extremes for the ethanol group. The results of these planaria was removed, resulting in the final group sizes mentioned above. Removing the high water and low ethanol data points led to a significant result ($F[2] = 10.00$, $p = <0.001$), with Bonferonni-adjusted post hoc analyses finding a very large ($t[24] = 4.37$, $p = <0.001$, $d = 2.12$) difference between the ethanol and water groups, with a slightly smaller, though still very large ($t[24] = 3.21$, $p = 0.011$, $d = 1.52$) difference between the ethanol and ethanol/naloxone group. No significant difference ($t[24 = –1.31$) was found between the H^2O and ethanol/naloxone groups. See Figure 7 for graphed results.

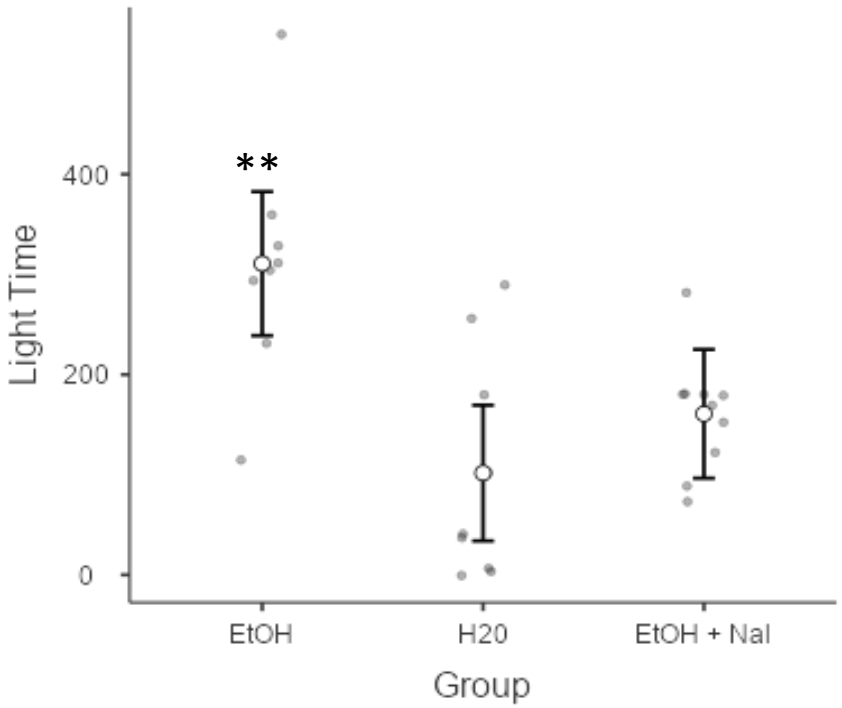

Figure 7: Means and 95% CI for each group in study 4. Total time for all three trials (maximum of 540 seconds attainable) on Y axis. Stars highlight significant difference from water only.

Discussion

Results of study 2 support the hypothesis that planaria can be operantly conditioned with ethanol in a spatial discrimination paradigm, while results of study 3 support the hypothesis that the mu-opioid receptor antagonist effectively interferes with ethanol-based reinforcement. The findings of study 4 support the hypothesis that naloxone attenuates the anxiolytic-like effects of ethanol in a test of avoidance behaviour.

This research is the first ever demonstration of operant conditioning in planaria with an addictive drug of any kind. The confirmation that planaria can indeed be trained to self-administer an addictive substance in a spatial discrimination paradigm is a significant discovery, implications for both our understanding of the evolutionary mechanisms supporting behaviour and the development of behavioural assays to explore the neural underpinnings of drug reinforcement. Since self-administration paradigms are considered the "gold standard" (Haney & Spealman, 2008) in drug research, this pioneering study has proven that planaria are suitable candidates to study the mechanisms by which drugs influence and modify behaviour. Additional to this significant discovery, the present results also show that the mu-opioid antagonist naloxone interferes with conditioning and the anxiolytic-like effects of ethanol. This implies that mu-opioid receptor activation is required for the reinforcing and anxiolytic-like effects of ethanol in planaria. Despite a scientifically unhealthy culture of many journals publishing only significant results – potentially exacerbated by researchers' unwillingness to submit these results – the results of these studies would have been equally important had the data shown the inverse pattern. Knowing the limits to applicability of specific animal models of drug addiction to human research is as much about knowing what cannot be utilised as it is knowing what can be. Should these results have indicated no reinforcing effect of ethanol in an operant paradigm and no effect of

naloxone in attenuating the anxiolytic-like effects of ethanol, understanding the useful limits of these species for drug research would have been a positive outcome in itself.

These results offer preliminary evidence that the same GABA-opioid-dopamine chain which has been identified in humans may be evolutionarily conserved from invertebrates such as planaria, up through the phylogenetic tree into higher order species. While these results certainly do not prove the link definitively, future research could further explore this possibility. Potential complementary studies might use a dopamine antagonist to compare against naloxone within a similar paradigm for ethanol or other drugs of abuse. The potential benefit of neurochemical assays on planaria pre- and post-conditioning may also reveal informative changes in different neurotransmitter systems caused by drug administration. It is also important to recognise both the relevance, but also the lack of direct generalisability of this research to human issues concerning drug abuse and addiction. There is still insufficient evidence to conclude that mu-opioid receptors are integral to the effect of ethanol reinforcement in planaria – more research is necessary.

Despite the caveats mentioned above and the need to curb premature excitement, many thought-provoking questions arise from this study. For what reason might the chain linking GABA, endogenous opioids and dopaminergic systems be evolutionarily conserved? Ultimately, some of the questions surrounding evolutionary conservation of any neurological system reach the heart of the "problem of psychology" (Henriques, 2011). This problem, broadly formulated, is that psychology lacks coherence as a unified scientific discipline. Other issues include attempts to consider living organisms in purely mechanistic ways, which is a perplexing approach when considering that uncertainty and dynamism have been recognised as key principles in physics for decades, a field which deals with ostensibly dead matter (Busch et al, 2007).

Evolution is, broadly speaking, the process of selecting characteristics amongst members of a species which best enable living organisms to adapt and thrive in an environment. According to William James (James, 1907), a philosopher and psychologist heavily influenced by the theories of Darwin, the process of evolution has equipped animals and humans to interpret and interact with the world at the level which they inhabit – effectively suggesting that different kinds of lifeforms inhabit different meta-environments. It is certainly accurate to say that the evolutionarily relevant aspects of the world are different for planaria and humans. For this reason, James considered evolutionary traits which are shared across species to be of paramount importance. From this perspective, uncovering the evolutionary reason for shared mechanisms of action, both behavioural and neurochemical, between planaria and humans is vital. One difficulty is that many theories of addiction in humans are not directly applicable to lower-order animals, especially outside the mammalian class.

A theory of human addiction which complements the data usually found in animal self-administration models, is the theory of addiction as learned behaviour (Lewis, 2018). This learning is based around a positive feedback loop, wherein drug use initiates neurological changes in memory and object recognition, or salience, which then alter general cognition and executive function. However, this theory also considers choice to be a vital element in human addiction (Lewis, 2017) and that the behaviour of people using drugs is not simply the result of neurotransmitter signalling. It seems difficult, if not impossible, to determine whether animals make, or are capable of making, conscious decisions in the manner defined by this theory. Other research has directly made the claim that addiction should be considered a form of implicit memory-based changes in cognition (Stacy & Wiers, 2006).

Additional related research in the field of third-generation cognitive science has attempted to explain the development of cognition from simple systems – evolutionarily old and basic brains – into the complex and multi-faceted systems present in humans (Vervaeke et al, 2012). This relatively new theory, involved simultaneously with psychology, artificial intelligence research and philosophy, rejects Cartesian dualism and proposes that the brain is the physical substrate for consciousness. Thus, the differences between animal and human mental processes stems not from a difference in kind, but a difference in degree. A key element of this theory is relevance realisation (Vervaeke & Ferraro, 2013) which the proponents argue is the fundamental purpose to all neurologically mediated functions in the animal kingdom. As a serious application of William James' philosophy and theory of pragmatism, relevance realisation is suggested to begin with unconscious, reactive processes, including those which might be present in all creatures possessing nerve cells. The unconscious elements of relevance realisation are also present in humans. Without (or at least before) conscious control, elements of the spine can initiate movement such as jerking a limb away from an intense source of heat such as a stove, with minimal contact and sensory information. This is an everyday example of an obviously adaptive mechanism, which is indeed mechanistic – holding one's hand to a hot stove requires exertion of executive function to override the normally unconscious reaction. While reactive nervous mechanisms may be the evolutionary genesis of relevance realisation (as evolutionary ancestors which lacked these preconscious abilities went extinct) they are certainly not the end.

With continued evolution, nerve cells develop to have greater diversification, specialisation, interconnectivity and react to a wider range of external stimuli as these become adaptively relevant (Vervaeke & Ferraro, 2013; Vervaeke et al, 2012). Complexified systems like the human nervous system are then emergent phenomena from evolutionary older brains, such as invertebrates (Sarnat & Netsky, 1985). While many animal models of

addiction seem to be almost mechanistic, the reinforcing effect of drugs on humans seems to also require an element of choice (Lewis, 2017), a choice planaria one could argue planaria are making on the basis of an internal representation of the drug (ethanol) as a goal, which may be representative of the emergent phenomena described by relevance realisation (Vervaeke & Ferraro, 2013; Vervaeke et al, 2012).

This cognitive science theory of relevance realisation and evolutionary emergence offers a strong framework from which to tie multiple fields together, connecting animal psychopharmacology research with human addiction research and neuroscience. Indeed, the neurological substrate for relevance realisation is adopted from many existing theories on learning (Vervaeke et al, 2013), such as neurotransmitter-mediated neurogenesis and memory formation (Kalueff & Nutt, 1996; Stagg et al, 2011; Wolf et al, 2004). The theory also helps to explain the commonality of spontaneous remission and other seemingly human-specific observations. Social isolation, for example, is a strong predictor of drug use and addiction in humans and other mammals (Christie, 2021; Hofford, 2021; Raz & Berger, 2010), likely due to increased sensitivity to dopamine (Yorgason et al, 2016), the neurotransmitter implicated in the reinforcing effect of most drugs of abuse (Barron et al, 2010). Psychological issues identified in humans as closely related to substance use, such as impaired delay of gratification (Kluwe-Schiavon et al, 2020; Oberlin et al, 2020) have also been associated with the same areas of the brain thought to be responsible for higher-order relevance realisation (Vervaek & Ferraro, 2013), possibly indicating some neurological relationship.

As complexification of neural systems increases, the notion of emergence may be necessary to account for less mechanistically explicable behaviour (Vervaeke et al, 2012). While some theorists belonging to the original school of behaviourism may discount uniquely human behaviours as simply more advanced versions of behaviour already observable in lower-order species, the recognition of emergent properties in what are indisputably more

neurologically advanced creatures achieves both greater explanatory power and as the late Viktor Frankl may say, “rehumanises psychology” (Frankl, 1955). Indeed, Frankl believed that reductionism, while immensely powerful, caused psychological research to constantly fall short of adequately examining uniquely human phenomena and was thus an insufficient method for its study (Frankl, 1966).

It is difficult to proclaim the direct relevance to humans of any finding in the animal kingdom – especially from a family so evolutionarily distant as flatworms – without first at least attempting to address the problem of psychology. While solving this problem was beyond the scope and not the aim of this research, the question of evolutionarily conservation of behaviour and neurological systems is important and inseparably linked to it, hence the brief attempt above to outline an empirically supported, however embryonic, unifying theory. With regards to the practical implications of the present findings, the results of these experiments highlight to role of mu-opioid receptor activation in mediating both the reinforcing and anxiolytic-like effect of ethanol in planaria. The significance of this in a pragmatic sense – that is, how should research and practice change because of it – lies in the possibility it affords scientists for testing new therapeutic interventions and assessing the probable mechanism of action, both behavioural and neurochemical.

It is well known that funding in the sciences is crucial for improving the quality and quantity of research (Ebadi & Schiffauerova, 2016), particularly in fields that may be considered most vital to public interests such as medical education research (Reed et al, 2007). In this context, it seems wise to conclude that any cost-saving measures which do not sacrifice the quality of research are of automatic relevance. For this reason alone, evidence suggesting that the role of planaria in developing pharmacotherapeutics can be safely expanded is indisputably a welcome discovery. While the extent to which planaria share functional neurochemical similarities with humans needs to be continually assessed, the

present study is a strong early indication for expanding this burgeoning field of research. To the extent that animal models are employed in psychopharmacology research, all worthwhile research, from both an economic and an ethical perspective, must be for a net benefit. Greater understanding of the shared factors mediating drug self-administration across the animal kingdom can ultimately only help to guide research aimed at reducing the suffering and burden on individuals and society caused by inappropriate and uncontrollable substance use.

In considering the human element of animal drug research, it is true that when adhered to, naloxone is an effective pharmacotherapeutic treatment for alcohol use disorders. However, in the majority of substance use disorders, individuals experience what is called "spontaneous remission" from the disorder (Cunningham et al, 2000). Some research has identified the rates of spontaneous remission to be over 77% among those with alcohol problems (Sobell et al, 1996). A more recent review of drug abuse remission conservatively estimated spontaneous remission prevalence of 18.2-26.2%, depending on the breadth of the definition (Walters, 2000). It is worth noting that this review included alcohol, tobacco and illicit drugs in the analysis. However, another study which also analysed the use of a variety of substances (Price et al, 2001) determined that spontaneous remission was the norm, rather than exception, though noting that individuals who did not achieve spontaneous remission had needs requiring serious consideration and support.

Because of the variability in success of pharmacotherapeutic methods in treating substance use disorders, as well as the prevalence of spontaneous remission and other reasons for cessation, other treatment methods need to be considered when approaching the human phenomenon. If individuals who maintain excessive drinking levels for long periods are usually capable of abstaining or drastically reducing their alcohol intake without treatment, then pharmacotherapies may not be of primary importance in alleviating the social and economic damage caused by alcohol consumption. Regardless, they remain a valuable tool in

assisting individuals who have difficulty in reducing their alcohol intake. Ultimately, research into treating substance use disorders must focus on methods which seem most widely applicable and effective to reduce suffering. Pharmacology-based therapies, while valuable and effective, are not the only viable solution.

How then might future studies with planaria make advances in determining the extent to which they share functional neurochemical systems with humans? Though difficult to execute, a future study with planaria could attempt to ascertain whether self-administration of a drug by planaria is affected by social isolation. Two experimental conditions could be maintained – those kept isolated and those kept in a group. Once baseline preferences are recorded, all planaria in the socially intermingled group could be separated into their side preferences, then have drug-associated arm choice measured. Daily mean choice rate could be compared between the isolated and intermingled groups. An obvious issue with this study would be the possibility of planaria reproducing in the social environment, but if successfully controlled for, this would be a tremendously important addition to the literature. Because sociality in the animal kingdom does not appear consistently until mammals, it seems unlikely to be a strong predictor of behaviour. Nonetheless, it remains a potentially important area to investigate in determining how well the planaria model of addiction can be generalised to higher-order animals.

A straightforward methodological addition for future studies would be to include the speed of locomotion and choice. This would provide a measure of increased drug salience and increased “motivation” (or at least the allocation of evolutionarily valuable metabolic resources) to obtain it. Speed of Y-maze arm choice could be measured across baseline tests. The time before a choice in drug-paired tests could be measured and the averaged times of each planaria compared. Whether these time measurements are compared across every day or only days at which planaria show an increased preference for the drug-paired arm which is

statistically significant would be up to the individual researchers. Time and drug choice rate could be analysed via correlation within a specific condition to assess whether changes in drug choice are accompanied by a simultaneous increase in speed. Another option would be to compare across groups with a method such as ANOVA, to see if differences in speed differ significantly across dependent variable conditions or between baseline and significantly higher drug choice experimental days.

A result with several interesting possible methodological additions was that of study 4. While ethanol alone exhibited the same anxiolytic-like response found previously with planaria in a light/dark paradigm (Zewde et al, 2018), there was no significant difference between the water and water/naloxone groups. This raises the question as to why naloxone would block what may have been hypothetically a purely GABA-reliant anxiolytic response. A possible study which would help explain this would be the application of an opiate such as morphine in the same lightbox paradigm. If a potent opiate such as morphine produces a greater tolerance for light than ethanol, this may indicate that anxiolytic-like behaviour in planaria is mediated more strongly by opioids than GABA. This would explain why naloxone appears to attenuate the increase in light tolerance which our results indicate.

Although not statistically significant, the mean time spent in light for the ethanol/naloxone group in study 4 was greater than the water-only group, possibly indicating a minor anxiolytic-like response. Utilising different concentrations of naloxone in future studies of this nature may help to determine whether the effect of ethanol is completely block, or merely limited by mu-opioid antagonists. This may also help to ascertain the degree to which GABA alone is responsible for anxiolytic-like effects.

The dose-response study was afflicted by the issue of a large proportion of planaria dying, but this was effectively dealt with through changes in handling and storage between

experiments in subsequent studies, leading to overall satisfactory results. Primarily, these studies were limited by using only one dose of ethanol to produce reinforcement and anxiolytic-like behaviour, as well as only a single dose of naloxone for the purposes of interfering with these effects. Ideally, multiple concentrations of both ethanol and naloxone could be applied, to establish dose-specific changes in behaviour. However, these options were not feasible for the present research team, as such rigour would have required multiple more experiments or more groups within experiments.

The behavioural paradigms used also have limitations. One paradigm was to measure reinforcing efficacy (the Y-maze) and converging evidence could have been sought using additional behaviours (such as conditioned place preference to measure ethanol-induced reward). Similarly, alternative paradigms could have been used to evaluate fear-like responses in planaria. Akin to the limitations mentioned previously, including these additional paradigms would have added weight to the conclusions reached on this thesis but it would have resulted in the addition of a large number of additional experiments which could, of course, be performed in the future.

Despite the limitations of the present research, these experiments offer convincing preliminary evidence of important similarities between planaria and humans in the neurotransmitter-based processes of ethanol reinforcement. Thus, planaria may represent a cheap, effective and simple model system to screen for new drugs designed to reduce ethanol consumption. While rodents have historically been allocated this role of early, proof-of-concept research into novel pharmacology treatments, planaria offer significant benefits. The major advantages of lower costs, both initially and in upkeep, few restrictions around ethical practice and reduced space required to house and test the animals make them a remarkably appealing animal model.

Because of their limited behavioural indicators of addiction and significant differences in physiology, planaria are certainly not poised to completely replace rodents in drug research, but their use is a step towards the consideration and implementation of alternative research approaches which do not use animals. One of the three guiding principles of the ethical, humane and responsible use of animals in scientific research is indeed "replacement" where possible. Benefits exist to the behavioural simplicity of planaria also, including the ease of creating and assessing paradigms with less behavioural ambiguity and variability. Compared to rodents, planaria may offer a more convenient entry point for studies with lower budgets, fewer researchers, less appropriate facilities or inadequate space for rodent research.

Declarations

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Competing interests

The author declares no competing interests.

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Appendices