

Planaria as a model for the effects of the co-use of alcohol and nicotine

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Abstract

Poly drug abuse is the abuse of more than one drug, often simultaneously, and is common in many drug-abusers in real-life conditions (Raffa, 2008). The co-use of alcohol and cigarettes is especially prevalent among heavy drinkers such as those diagnosed with alcoholism or alcohol abuse (Bobo & Husten, 2000). The purpose of this study is to gain further insight into the potential mechanisms involved in the poly-drug abuse of alcohol and cigarettes by observing the withdrawal effects of each individual drug versus the combined drugs in the planarian flatworm. Planarian locomotor velocity (pLMV) and atypical behaviours are the behavioural paradigms used in this experiment to quantify the withdrawal effects observed. Results reveal a complex relationship between alcohol and nicotine with interactions between concentration and exposure time to influence the poly-drug relationship.

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Co-use of alcohol and cigarettes

Poly drug abuse is the abuse of more than one drug, often simultaneously, and it occurs in many drug-abusers in real-life conditions (Raffa, 2008). The purpose of this study was to determine whether a significant difference exists between single drug use and poly-drug use as is suggested in the book *Planaria: A model for drug action and abuse* (Raffa, 2008).

The co-use of alcohol and cigarettes is especially prevalent among heavy drinkers such as those diagnosed with alcoholism or alcohol abuse (Bobo and Husten, 2000). According to Patten and colleagues (1995) 95% of alcoholics smoke cigarettes with approximately 70% of these individuals being considered heavy smokers (smoking an average of more than one pack a day) (Collins & Marks, 1995). In 1996, Hurt et al. reported the cigarette use of in-patient alcoholics to be 75%.

Moreover, based on correlative studies, it was found that individuals who were alcohol dependent were three times more likely to be cigarette smokers than individuals of the general population, and those who were dependent on cigarettes were four times more likely than the general population to be dependent on alcohol (Grant et al., 2004). Thus, individuals who smoked were more likely to drink and vice versa.

When it comes to poly-drug abuse, understanding the interaction between the effects of the co-use of alcohol and cigarettes on the brain is essential and would have important implications in various healthcare fields (NIAAA, 2007).

However, actually discovering the separate mechanisms involved in creating the combined effect of the drugs has proven to be rather difficult as their mechanisms involve some of the same receptors and their co-use is quite common (NIAAA, 2007).

Thus, the purpose of this study is essentially to tease apart and gain further insight into the mechanism involved in the poly-drug abuse of alcohol and cigarettes by studying the behavioural effects each drug exerts on a planarian model individually and then combined. In order to attempt this understanding of the mechanisms involved in the interaction between alcohol and tobacco, it is first necessary to understand each drug, as well as the neurobiology involved in the consumption of each drug, individually.

Neurobiology of alcohol dependence

Alcohol consumption is the third leading cause of preventable deaths worldwide (Spanagel et al., 2013). The mechanism of addiction to alcohol, from the initial intoxication to the initiation of the behaviour that allows the individual to maintain the excessive drinking habit, while promoting relapse during abstinence, occurs primarily in the brain (Lovinger, 2008).

Communication in the brain occurs between neurons, which transfer information using chemical messengers known as neurotransmitters (Yamashita, 1998). Neurotransmitters are released by one neuron and bound to another by specialized protein receptors (Yamashita, 1998). Alcohol works by reducing the excitatory effects of glutamate on the NMDA receptor subtype and increasing the inhibitory actions of gamma-aminobutyric acid (GABA) at the GABA_A receptor

(Yamashita, 1998). In this respect, alcohol is commonly referred to as a depressant due to its mainly inhibitory action on the brain (Yamashita, 1998). Alcohol does not only act on these neurotransmitters however (Yamashita, 1998). It actually has a fairly widespread set of targets in the brain and is believed to also play a role on other neurotransmitters such as dopamine, serotonin (5-HT), acetylcholine and opioid peptides (Yamashita, 1998). These acute effects of alcohol consumption differ greatly from the chronic effects of alcohol abuse (Yamashita, 1998).

Ethanol acts as a positive reinforce (Weiss and Porrino, 2002). Like all drugs it causes the release of dopamine in the ventral tegmental area of the mesocorticolimbic reward pathway (Weiss and Porrino, 2002), which then projects to the nucleus accumbens, amygdala and frontal cortex (Markou, 2008). While most other abused drugs have been shown to have direct actions at dopamine synapses, alcohol is believed to act on dopamine synapses indirectly through GABA (Weiss and Porrino, 2002). The actual mechanism for this process is still unknown (Weiss and Porrino, 2002).

Both neuroadaptation and reinforcement contribute to the development of the drug dependence by underlying the acute response in addition to the establishment of the chronic craving characteristic of addictions (NIAAA, 2000). Neuroadaptation is a term used to describe the compensatory adjustments the brain makes in order to normally function despite the presence of the abused drugs (NIAAA, 2000). As seen in chronic alcohol exposure, this neuroadaptive change may be a permanent change and is a contributing factor to the relapse

seen in drug addictions (Weiss and Porrino, 2002). Adaptations such as dopamine hypofunction in the mesolimbic pathway occur as a means of countering sustained stimulation of the system by alcohol (Weiss and Porrino, 2002). Reinforcement occurs when a rewarding stimulus (for example drugs or any rewarding behavior), or the relief of an unpleasant state, causes the increased probability of a behavioural response (NIAAA, 2000). The hypofunction of dopamine that occurs as a result of the neuroadaptations are responsible for the reinforcement of the alcohol dependence (Weiss and Porrino, 2002). The mesolimbic dopamine hypofunction is significant enough to cause the maintenance of the addiction by promoting the intake of alcohol as a compensatory measure for the decreased efficacy of dopamine release (Weiss and Porrino, 2002). It also motivates the resumption of drinking in cases of cessation to reverse the dopamine deficits (Weiss and Porrino, 2002). Thus, the withdrawal effects experienced promote the addicted user to resume the addictive behaviour (Weiss and Porrino, 2002).

Neurobiology of nicotine dependence

Nicotine has been identified as the main addictive substance in tobacco (Markou, 2008) and is responsible for the resultant stimulant effects, reinforcement and dependence that result from tobacco use (Kopnisky and Hyman, 2002). Addiction to nicotine is the most prevalent form of substance abuse (Markou, 2008) and the leading cause of preventable disease, disability and death in the United States of America (CDC, 2014). A one-time user of tobacco smoke has an estimated 33% probability of becoming tobacco-

dependent (Anthony, 2002). In comparison to alcohol, which has an estimated probability of drug dependence among first-time alcohol consumers of only 15%, and other addictive substances such as heroin, cocaine and cannabis, which have probabilities of 23%, 17%, and 9% respectively, tobacco remains the drug with the highest dependency probability (Anthony, 2002). It is powerfully reinforcing even in the absence of subjective euphoria (Kopnisky and Hyman, 2002).

The nicotine in cigarettes works by imitating the natural effects of the neurotransmitter acetylcholine by binding to its nicotinic receptor. Many nicotinic receptors are situated on presynaptic terminals and are responsible for modulating neurotransmitter release (Wonnacott, 1997). Nevertheless, nicotinic receptors may also be found at somatodendritic, axonal and postsynaptic sites (Markou, 2008). This wide distribution of nicotinic receptors means that nicotine stimulates the release of most neurotransmitters in the brain (McGehee and Role, 1995). As a result, various neurotransmitter systems are involved in the rewarding effects of nicotine and the neuroadaptations that occur in response to chronic nicotine exposure from which dependence and withdrawal responses arise (Markou, 2008).

Exogenously administered nicotine, such as in cigarette smoking, directly increases dopamine transmission by acting on specific nicotinic acetylcholine receptors within the ventral tegmental area (Markou, 2008). Nicotine also excites nicotinic receptors on glutamatergic (excitatory) and GABA-releasing (inhibitory) terminals, resulting in a greater overall increase in dopamine levels (Markou,

2008). Chronic nicotine exposure leads to nicotine receptor desensitization, which results in the up-regulation of these receptors (Perry et al., 1999). Despite the change in number and function of nicotinic receptors, no neuroadaptations in GABA function have been found with the development of nicotine dependence.

The mesocorticolimbic pathway, which has already been discussed in pertinence to alcohol, is critically involved in several drugs of abuse, including nicotine abuse (Markou, 2008). Ample evidence implicates glutamate-GABA-dopamine-acetylcholine interactions, particularly in the ventral tegmental area, in several behavioural and affective responses to nicotine (Markou, 2008).

Other unidentified products in tobacco that are thought to contribute to cigarette addiction act by inhibiting monoamine oxidase B (MAO B) (Markou, 2008). MAO B is responsible for breaking down dopamine after its reuptake resulting in higher concentrations of dopamine in the reward circuit that contributes to the dependency (Markou, 2008). Nornicotine is also recognized to contribute to cigarette addiction (Markou, 2008).

Current knowledge on the interactions of alcohol and cigarettes

For many years it has been known that alcoholism and cigarette addiction very commonly co-occur (Davis and de Fiebre, 2006). In 1860, Reverend George Trask even commented on how uncommon it was to know a drunkard who did not use tobacco (Trask, 1860). Even so, to this day, relatively little is actually known about the neurobiological mechanisms underlying the co-abuse of both alcohol and cigarettes (Davis and de Fiebre, 2006). As reviewed above, both drugs appear to act on many of the same neurotransmitters (nicotinic

acetylcholine receptors, GABA, glutamate), however the relationship and mechanism of the combined interaction is still largely unknown. This issue is even recognized as an underfunded and understudied area of research (Davis and de Fiebre, 2006). Efforts to shed some light on the type of interaction that occurs at the receptor level when alcohol and nicotine (the main addictive ingredient in cigarettes) are co-abused will be made by observing specific behaviours in planaria.

Furthermore, the elucidation of the type of interaction that occurs between drugs used in polydrug abuse is quite significant as well (Raffa et al., 2006). In the past, a quantifiable experimental method to establish this type of analysis was difficult. However, Raffa defined some easily quantifiable withdrawal effects displayed in planaria using cocaine, which have allowed the demonstration of synergistic or antagonistic drug interactions to be possible (Raffa et al., 2006). Raffa, Stagliano and Tallarida (2006) were able to demonstrate synergistic effects of the cocaine-canniboid combination in planaria using pLMV, establishing for the first time the quantification of this synergy. Thus, the cocaine-canniboid combination elicited greater effects in pLMV than either drug on it's own. Similarly, this study aims to establish quantifiable results using the behavioural paradigms of pLMV and atypical behaviours developed by Raffa to show the relationship between alcohol and nicotine.

Using planaria as a model for poly-drug abuse

Planaria have been one of the best-characterized animal models for research in molecular biology and regeneration (Pagan et al., 2012), and have

been recently rediscovered as great models in neuropharmacology research (Raffa, 2008). Planarians are an attractive model for studying the neurochemistry of the pathways involved in poly-drug abuse and addiction due to their primitive but vertebrate-like nervous system, mammalian-like neurotransmitters and their simple pharmacokinetic interactions compared to other animal models (Raffa, 2008). Most importantly, they respond with specific behaviors to selective ligands (Venturini et al., 1989).

Planaria are simple free-living flatworms and are known as the first metazoan and multicellular animal to not only possess a central nervous system but also have the same body plan template as seen in all vertebrates: a bilateral symmetry, three axes and cephalization (Sarnat and Netsky, 2002). Most planaria are less than a centimetre in length, and in spite of this, have a brain to body weight ratio that is similar to that of the rat (another commonly used animal model in experimental research) (Sarnat and Netsky, 2002).

Planaria have dorsal and ventral surfaces and a head and tail, as well as specialized sense organs and a primitive “brain”, which most authors refer to as “cephalic ganglion” (Sarnat and Netsky, 2002). The planaria’s cephalic ganglion is bilobar and contains many commissural fibres that interconnect both halves. They have motor and sensory nerves, which innervate both body muscles and peripheral structures respectively. In addition, they have a pair of neural cords that include primary motor neurons, sensory neurons and interneurons, essentially representing a simple version of the vertebrate spinal cord. Despite the simplicity of the planaria, its neurons surprisingly more closely relate to those

of vertebrates than invertebrates (multipolar shape, dendritic arborizations, a single axon, expression of similar vertebrate neural proteins as well as spontaneously generated electrical activity).

Planaria possess nearly all of the major classical neurotransmitters present in mammals. Evidence of serotonin (5-HT), catecholamines, acetylcholine, gamma-amino butyric acid (GABA), excitatory amino acids (ie: glutamate and aspartate) neurotransmitter systems, as well as an enkephalinergic neurotransmitter system, has been provided in planaria (Buttarelli, 2008). The enkephalin receptor in planaria is equivalent to the mammalian k-opioid receptor in humans (Raffa et al., 2006).

The usage of planaria allows for a much simpler look at the interaction between alcohol and tobacco at the receptor level barring any of the pharmacokinetics that would occur in more sophisticated animal models (ie: vertebrates such as the rat) (Raffa, 2008). Importantly, since planaria lack a blood-brain barrier and have such stereotyped and specific responses, the discernment of the complex interactions of alcohol and nicotine should be less affected by any intrinsic impedances one would encounter with mammalian animal models, while still revealing human applicable and pertinent information (Murugan and Persinger, 2014).

Current study

Purpose

The main target of this study was to determine whether there is a difference between the effects of single drug use/abuse and poly-drug

use/abuse. Addiction research in general is such an important area of study because it allows for a greater understanding of addiction. As the science of addiction becomes more and more clear, we are able to find and develop more successful ways to treat it. The abuse and addiction of alcohol, nicotine and illicit and prescription drugs costs the United States over \$700 billion each year with increased health care costs, crime and losses in workplace productivity (National drug intelligence centre, 2011; Rehm, Mathers, Popova, Thavorncharoensap, Teerawattananon, & Patra, 2009; CDC, 2014). The importance of studying poly drug abuse lies in the fact that in the real world, most addicts are addicted to more than one drug and therefore partake in poly-drug abuse (Raffa, 2008).

The decision to focus on alcohol and nicotine is largely due to the massive global impacts of the abuse of these substances and their frequent co-usage. As previously stated, alcohol consumption is the third leading cause of preventable deaths worldwide (Spanagel et al., 2013). 3.3 million deaths, which accounts for 5.9% of all global deaths, were attributable to alcohol consumption in 2012 (World Health Organization, 2014). Alcohol consumption is a contributing factor to over 200 diseases such as alcohol dependence, liver cirrhosis, cancers and injuries (WHO, 2014). It is the primary risk factor for premature death in persons between 15 and 49 years of age (Lim, Vos, Flaxman, et.al. 2012). In the United states, 16.6 million persons over the age of 18 or 7% of adults suffered from an alcohol use disorder in 2013 (SAMHSA, 2013). It is also noteworthy to note that in 2012 7.5 million or 7.5% of children younger than 18 years of ages lived with a

parent suffering from an alcohol use disorder (SAMHSA, 2012). Children in these homes are put at greater risk for depression, anxiety disorders, cognitive and verbal skill problems, and parental abuse or neglect (SAMHSA, 2012). They are also four times more likely to develop alcohol use disorders than other children (SAMHSA, 2012). The economic burden of alcohol misuse was in the range of \$223.5 billion dollars in the United States alone in 2006 (CDC, 2014). These costs are mostly a result of the loss of productivity in the workplace, health care expenses, law enforcement expenses and motor vehicle crash costs from impaired driving which accounted for 72%, 11%, 9% and 6% of the total costs respectively (CDC, 2014).

More than 480,000 premature deaths, or 1 in roughly every 5 deaths, each year in the United States occur as a result of cigarette smoking making tobacco use the leading cause of preventable deaths in the United States (NIH, 2014). It is also the leading cause of disease and disability with 30 sufferers for every one person who dies from smoking (CDC, 2014). 33% of all cancers, including 90 percent of lung cancers are attributable to cigarette smoking (CDC, 2004). Nicotine is the addictive ingredient in cigarette smoke and only indirectly contributes to cancer by causing the addiction to the tobacco cigarette, which contains a complex mixture of known carcinogens (NIH, 2014). The average life expectancy of a cigarette smoker is 10 years less than the life expectancy of a non-smoker (NIH, 2014). Tobacco use also has serious impacts directed at non-users through second-hand smoke such as increased risk for disease from the exhaled smoke, as well as the smoke given off of the burning end of the cigarette

(NIH, 2014). Children in homes with smoking parents are more likely to become smokers themselves (NIH, 2014).

Research into the co-use and co-addiction of nicotine and alcohol, given the prevalence of each and the economic burden associated with them, is critical (Li, Volkow, Baler, and Egli, 2006). With up to 80 percent of alcohol dependent individuals smoking regularly, and the consistency of epidemiological studies in showing that drinkers tend to smoke and vice versa, the poly-drug abuse or co-usage of alcohol and nicotine is the ideal case and candidates to begin this line of research with (Li et. al., 2006). Particular regard to their interactions is essential as effective addiction treatment and prevention efforts in the co-usage could be unintentionally hindered while this is still unclear (Li et. al., 2006). Steps towards a thorough understanding of the mechanisms behind their interaction are crucial in eventually developing interventions that could positively impact overall public health (Li et. al., 2006). The current understanding of the neurobiology involved in the use of each drug has provided insight into some overlapping pathways influenced by each drug, an example being the reinforcing effects of the drugs by enhancing dopamine in the nucleus accumbens (Li et. al., 2006). Although separate mechanisms are responsible for the achievement of these effects, it appears that the effects are additive in their rewarding properties and thus point to a severely complex relationship between alcohol and nicotine (Li et. al., 2006).

Dependent variables

Two different behavioural paradigms were used as dependent variables: planaria locomotor velocity and atypical behaviours.

Planaria locomotor velocity (pLMV)

Planaria locomotor velocity is an easily quantifiable behavioural response that has been used to study the effects of a variety of drugs (Raffa, 2008).

Planaria locomotor velocity (pLMV) is a behavioural paradigm based on the counting of the number of gridlines crossed by planaria placed individually into a clear plastic petri dish over gridline paper. This simple, yet effective method was initially described by Raffa et al. in 2000 and subsequently applied as a particularly suitable model for investigating the effects of addictive drugs in planaria.

Knowing that abstinence-induced withdrawal behaviours are used as common experimental paradigms for the assessment of physical dependence in mammals (Grimm et al., 2001), Raffa and Valdez performed an experiment to validate the pLMV behaviour paradigm (Raffa and Valdez, 2001). Cocaine experienced planaria placed into cocaine-free water elicited an abstinence-induced reduction in pLMV. The magnitude of the effect was found to be dose-dependent (related to exposure concentration), and was not elicited when cocaine experienced planaria were transferred into water containing cocaine.

Atypical behaviours

Another behavioural paradigm developed and proven to be effective by Raffa and Desai in a study looking specifically for cocaine withdrawal 'signs' in

planaria is known as atypical behaviours (Raffa and Desai, 2004). The researchers transferred cocaine-experienced planaria into cocaine-naïve water and the following unusual behaviours were observed: headbopping, squirming, clinging, headswing, tailtwist and corkscrew movements. These atypical behaviors were not observed when cocaine-experienced planaria were transferred into cocaine-containing water (Raffa and Desai, 2004). These were the first quantification of direct evidence of withdrawal phenomenon and behaviours in planaria (Raffa and Desai, 2004).

The atypical behaviours are also of significant importance since the behaviours are cues and aid in providing knowledge of the receptors involved in the drug mechanism. Venturini and colleagues, discovered screw-like (corkscrew) behaviours corresponded to dopamine D1 receptors (Venturini et.al., 1989) and later they were found to also correspond to k-opioid receptors (Raffa et al., 2003). Additionally, C-like curling was found to correspond to dopamine D2 (Venturini et al., 1989) and glutamate receptors (Rawls, 2011).

Independent variables

The first independent variable was the drug type. The entire purpose of the study is to determine whether the effects of poly-drug use differ from the use of single drugs. Therefore, planaria were placed in the single drug group and either treated with alcohol or nicotine or they were placed into the poly-drug group and treated with alcohol and nicotine combined.

The second independent variable was concentration. Drugs at different concentrations have different effects; therefore it was critical to look at how

differences in concentration might affect the behavioural paradigms being measured.

The third independent variable was exposure time. It is well known that drugs have different acute and long-term effects, therefore exploring the effects of poly-drug versus single drug use in terms of an acute and long-term model naturally made sense.

Hypotheses

There was an expected decrease in locomotion (pLMV) and general increase in the number of certain observed atypical behaviours in all the drug groups in comparison to control groups.

The main hypothesis surrounds the purpose of the study which is to determine whether or not a difference exist between the effects of poly-drug use and single drug-use, specifically between alcohol and nicotine. For planaria locomotor velocity, there was an expected significant difference between the alcohol and the combined drug group. Similarly, a significant difference was expected between the nicotine and the combined drug group. A synergistic effect was expected where the effect of the combined drug group would be significantly higher (thus planaria velocity would be more negatively affected), in comparison to either of the single drug groups.

A difference in the atypical behaviours observed for each of the drug types was expected since all the behaviours represents the binding of a specific receptor. There was an expected significant difference in the behaviours elicited

between the single drug groups and between the single and the poly drug groups.

There was an expected increase in the effects of each drug as the concentration increased. Thus, for planaria locomotor velocity, a decrease in locomotion as the concentration increased was expected. For the atypical behaviours, there was also an expected decrease in behaviours displayed as the concentration increased, based on the assumption that as there is less movement there is less likelihood of displaying atypical behaviours.

There was an expected significant difference between 1-hour and 24-hour exposure times, which represented acute (short-term) and long-term effects in the planaria. Drugs have been shown to have different short and long-term effects as neuroadaptations occur in the brain, causing dependency and addiction (NIAAA, 2000). Long-term (24-hour) exposure was expected to cause significant decreases in locomotion due to significant damage to the system. However, it was also plausible that the system adapted and developed a tolerance causing increased locomotion. For the atypical behaviours there was an expected difference in frequency between behaviours, with some behaviours becoming more or less frequent in the acute versus long-term groups. Logically speaking, this would make sense as some of the initial receptors activated in the acute effects may be less activated in the long-term effects due to neuroadaptations (Weiss and Porrino, 2002).

Materials and methods

Planarian maintenance

Brown planaria obtained from Carolina Biological Supply were acclimated to local laboratory conditions, housed in darkness in spring water and utilized in experiments within 72 hours. The spring water was obtained from Feverham, Grey Country, Ontario in Canada. Ion contents of the spring water in ppm were HCO₃ 270, Ca 71, Mg 25, SO₄ 5.9, Cl 2.7, NO₃ 2.6 and Na 1.

Preliminary determination of drug concentration

Ethanol (alcohol) and nicotine were obtained and diluted in spring water to the desired molarity or concentrations:

- Control (0%);
- Nicotine (5mM, 1mM, 100µM, 30µM, 10µM, 1µM); and
- Ethanol (5%, 3%, 1%, 0.5%, 0.1%, 0.01%).

Planaria locomotor velocity (pLMV), a behavioural paradigm that measures locomotion by counting the number of gridlines crossed in a specific timeframe, was used to determine the dosage of each drug that was significantly different from the control ($p < 0.05$), without killing the planaria. These determined optimal concentrations were used to carry out the actual experiment:

- Control (0%);
- Nicotine (100µM, 30µM, 10µM, 1µM);
- Ethanol (1%, 0.5%, 0.1%, 0.01%); and
- Combined drugs (100µM+1%, 30µM+0.5%, 10µM+0.1%, 1µM+0.01%).

Procedure

The worms were removed from group containers and randomly assigned to a drug group and concentration. These worms were then housed individually into appropriately labeled 1.5 ml plastic conical Fisherbrand centrifuge tubes with flat-top snap caps containing the desired concentration of the selected drug (ethanol, nicotine, ethanol-nicotine combination or water as a control). By first removing the desired number of planaria from the group containers and subsequently placing them in a petri dish, and from there assigning them to their own individual tube, I was able to assure the random assignment of planaria.

Worms were exposed to the drug for one hour and then subsequently removed one at a time and placed in fresh spring water. A timer was immediately started and the number of squares crossed per minute for a 5-minute period was recorded cumulatively. During this same period, the number of observed atypical behaviours was recorded. This same procedure was repeated with an exposure time of 24 hours with a new group of drug-naïve planaria.

5 planaria per concentration (control or 0%, 100 μ M, 30 μ M, 10 μ M, 1 μ M, 1%, 0.5%, 0.1%, 0.01%, 100 μ M+1%, 30 μ M+0.5%, 10 μ M+0.1%, 1 μ M+0.01%), per drug type were used for each exposure time (1 hour vs. 24 hours) for a total of 3 trials. Therefore N=15 per concentration, per drug type. The total number of planaria used for this experiment was 390.

Locomotion: (pLMV)

As previously stated, planaria locomotor velocity (pLMV) has been demonstrated as an effective means of quantifying withdrawal effects in planaria

(Raffa and Valdez, 2001, Raffa et al., 2003). Locomotion was tested using this reliable technique.

Planaria were exposed to the selected drug, (water as a control, alcohol, nicotine or alcohol-nicotine combination), for one hour and subsequently transferred into drug-free water in a clear plastic petri dish. The petri dish was placed on top of a 0.5cm gridlines. The number of gridlines crossed per minute was cumulatively recorded over a five-minute period.

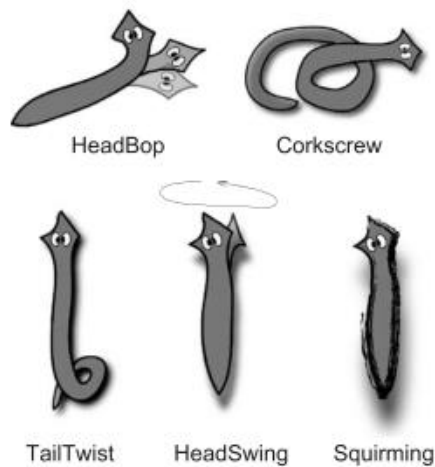
The exact same procedure was repeated but instead after a 24-hour drug exposure time.

Behaviour: atypical behaviours

In the same five-minute period, planaria the same planaria that had been exposed to the selected drug and concentration, (water as a control, alcohol, nicotine or alcohol-nicotine combination), for one hour and subsequently transferred into the drug-free water in a clear plastic petri dish were observed for any signs of the atypical behaviours defined below by Raffa and Desai (2004).

Definition of atypical behaviours (depicted in the figure below):

1. Headbob: nodding movement of head while moving forward
2. Corkscrew: spiral motion around long axis
3. Tail twist: tip of body twisted
4. Headswing: axial rotation of head while tail is anchored, 'helicopter' motion
5. Squirming: uncoordinated, jerky movements
6. Clinging: scrunching, typically intertwining with another planarian



Results

All analyses were conducted with SPSS, using an alpha of .05.

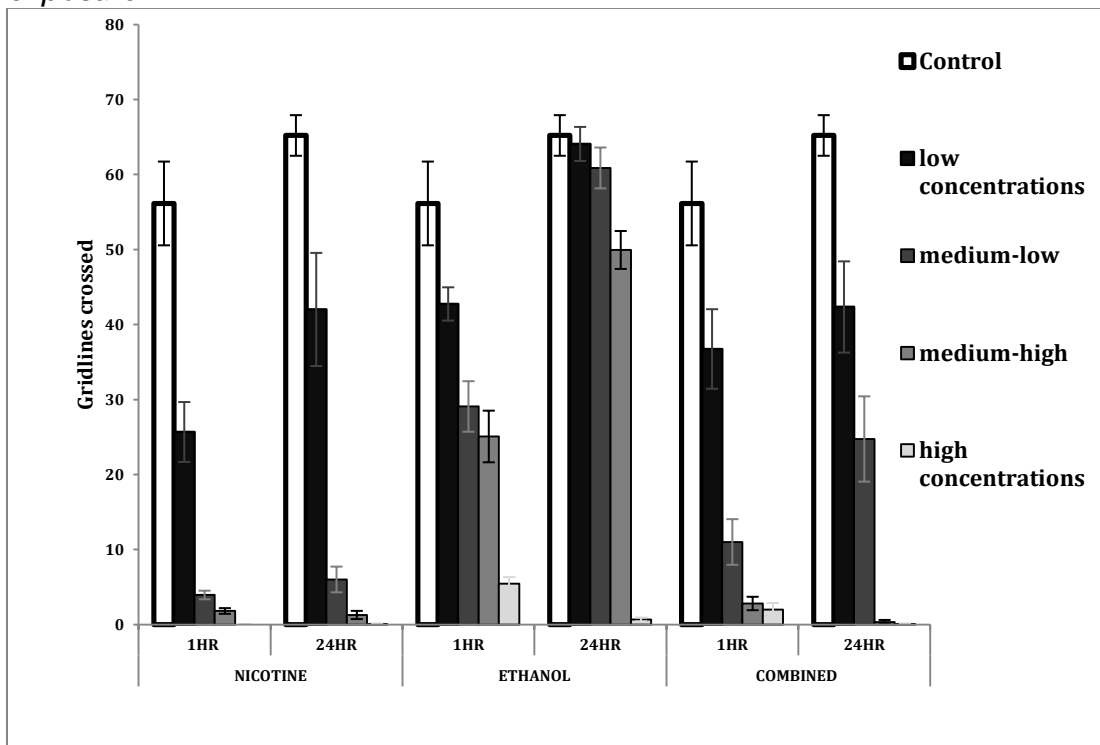
Planaria locomotor velocity

Planaria locomotor velocity (pLMV) over a 5-minute period was analysed using a three-way ANOVA. There was a significant interaction between drug type, concentration and exposure time on the number of gridlines crossed, $F(6, 364)=3.497$, $p<0.005$, $\eta^2=.055$. This significant interaction indicates that the drug types (alcohol, nicotine, and the alcohol and nicotine combined) were affected differently by concentration and exposure time. As expected, analyses revealed that all drug groups, (with the exception of the lowest .01% alcohol group) had significantly ($p<.05$) decreased locomotion (pLMV) compared to controls. Also, controls showed generally lowered or no significant differences in the number of observed atypical behaviours compared to all the drug groups.

Simple effects of drug type

Analysis of the simple effects of drug type revealed significant differences ($p < 0.001$) in the 1 hour and 24 hour exposure times of medium-high, medium-low and low concentrations. There was no significance in the highest concentration for either 1 hour exposure, ($F(2,364) = .73$, $p = .481$) nor the 24-hour exposure time, ($F(2,364) = .012$, $p = .989$).

Figure 1.
Number of gridlines crossed ($pLMV$) in terms of drug type, concentration and exposure.



At 1-hour exposure, the medium-high and medium-low concentrations showed significant ($p < 0.001$) decreases in the mean gridlines crossed between alcohol and the combined alcohol and nicotine groups (Figure 1). An average of 23.3 fewer gridlines were crossed for the medium-high concentration and 18.1 fewer for the medium low concentration by planarian exposed for one hour to

both alcohol and nicotine than those exposed to solely alcohol for the hour. No significance was found between nicotine and the combined group at these concentrations. However, at low concentrations there was a significant ($p < 0.001$) decrease in the locomotion of nicotine-treated versus the combined alcohol and nicotine treated planarian. There was no significance at the low concentration between alcohol and the combined group.

At 24-hour exposure, the medium-**high** concentration continued to show significant differences ($p < 0.001$) in the mean gridlines crossed between the alcohol and the combined alcohol and nicotine groups with an average of 43.6 fewer gridlines crossed in the combined drug group. Unlike the 1 hour-exposure, the medium-**low** concentration showed significance ($p < 0.001$) between all the drug types. Nicotine had the smallest mean of gridlines crossed, with a mean increase of 18.7 gridlines crossed for the combined drug group, while ethanol showed a significant increase of 36.1 gridlines crossed from the mean gridlines crossed of the combined group. The low concentration, contrary to the 1-hour exposure results, showed significant increases in the ethanol group's locomotion in comparison to the combined group's locomotion.

Simple effects of concentration

Analysis of the simple effects of concentration revealed significant differences ($p < .001$) in the 1-hour and 24-hour exposure times for each drug type (nicotine, alcohol, combined). As the concentration increased, planaria locomotor velocity (or the number of gridlines crossed), decreased.

Nicotine showed significant differences ($p < .001$) between the low concentration and all the other concentrations at both 1-hour and 24-hour exposures (Figure 2 and 3). Planaria treated with the low concentration of nicotine had more movement than in the other three higher concentrations.

Figure 2.
Number of gridlines crossed cumulatively over 5-minutes after 1-hour exposure in terms of concentration for nicotine.

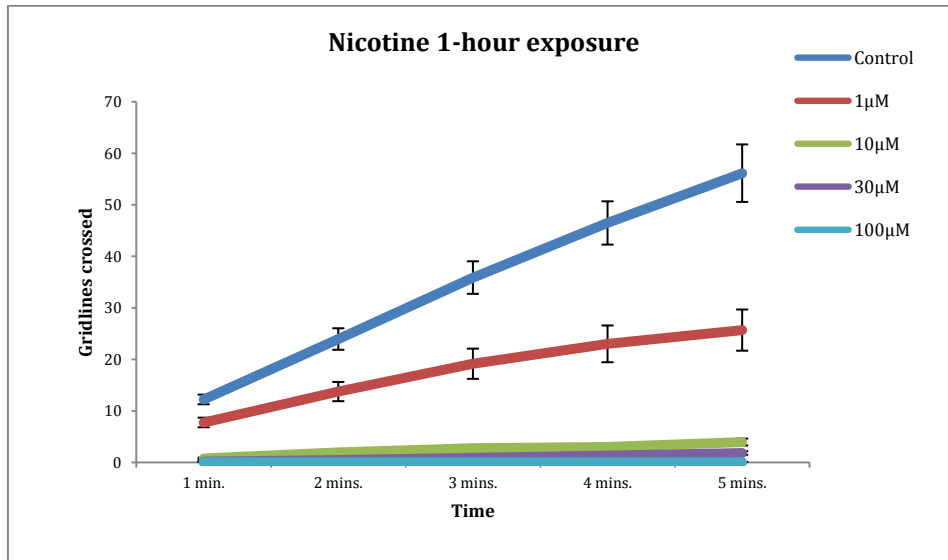
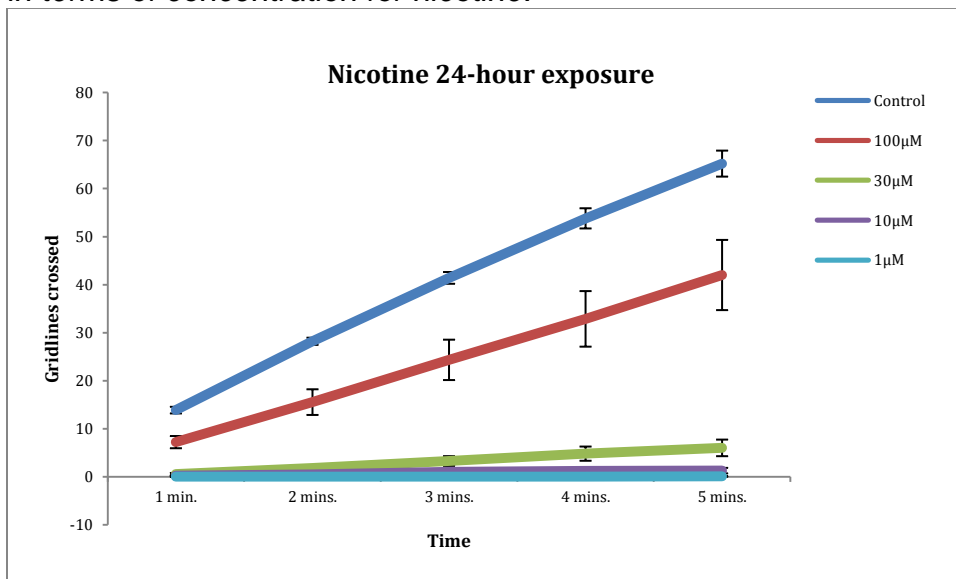


Figure 3.
Number of gridlines crossed cumulatively over 5-minutes after 24-hour exposure in terms of concentration for nicotine.



At 1-hour exposure (Figure 4), ethanol displayed significant differences ($p < .005$) between all concentrations with the exception of the medium-high and medium-low concentrations which were not significantly different ($p = .382$). At 24-hour exposure (Figure 5), ethanol displayed significant differences ($p < .001$) between all concentrations with the exception of a non-significant difference between the medium-low and low concentration at this exposure ($p = .484$).

Figure 4.
Number of gridlines crossed cumulatively over 5-minutes after 1-hour exposure in terms of concentration for ethanol.

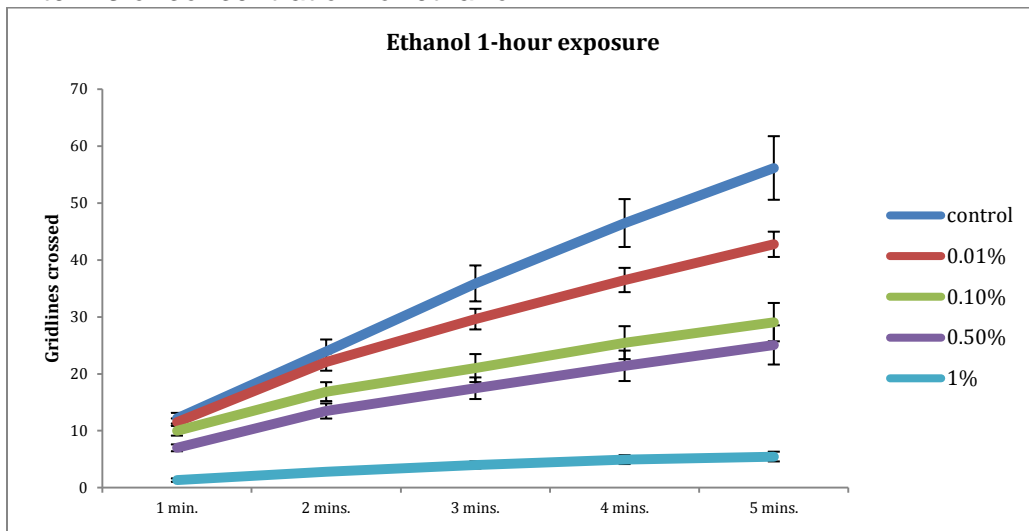
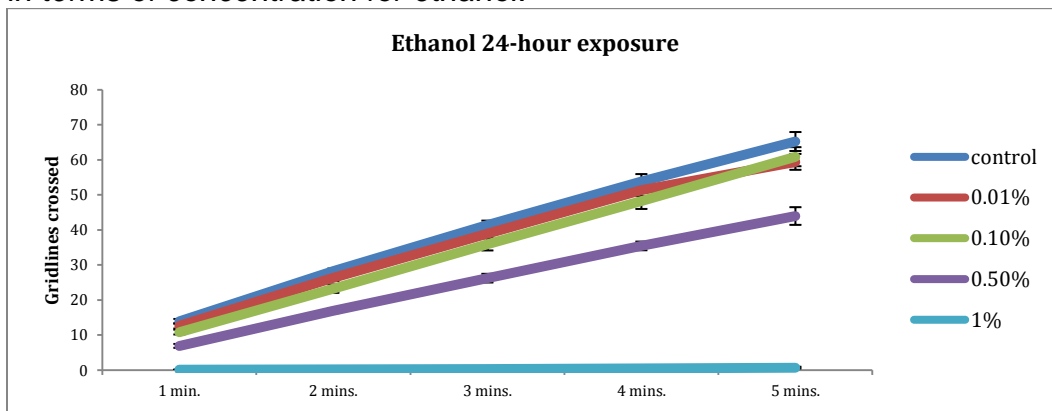


Figure 5.
Number of gridlines crossed cumulatively over 5-minutes after 24-hour exposure in terms of concentration for ethanol.



At 1-hour exposure (Figure 6), the combined ethanol and nicotine displayed a significant difference ($p < .001$) in the mean number of gridlines crossed between the lowest concentration and all the other concentrations. There was also a significant difference ($p = .05$) between the high and medium-low concentrations. After 24 hours of exposure (Figure 7), the pattern changed to display significant differences ($p < .001$) in the mean gridlines crossed between all concentrations with the exception of the high and medium-high concentrations ($p = .953$).

Figure 6.
Number of gridlines crossed cumulatively over 5-minutes after 1-hour exposure in terms of concentration for combined alcohol and nicotine.

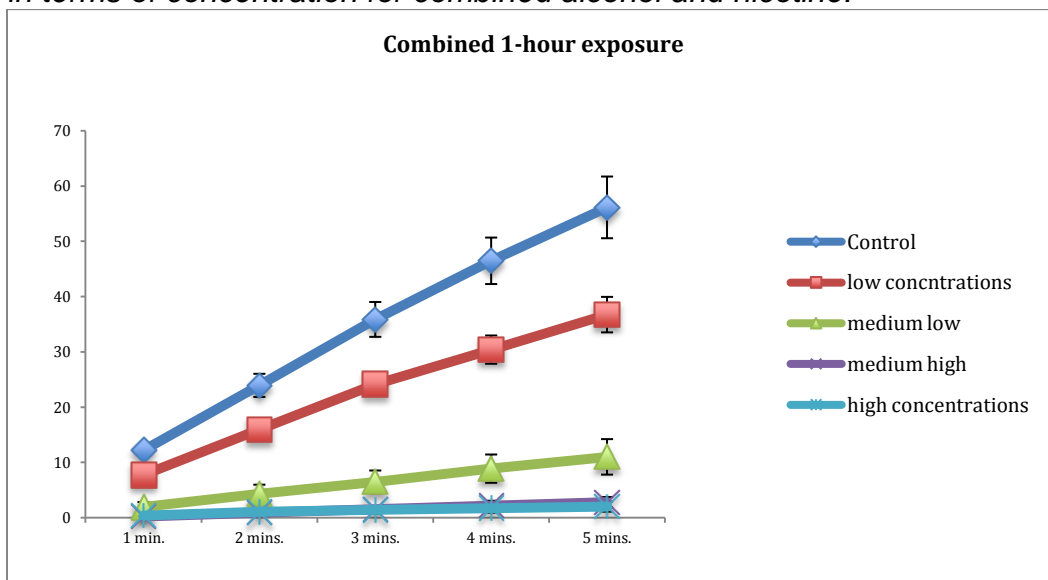
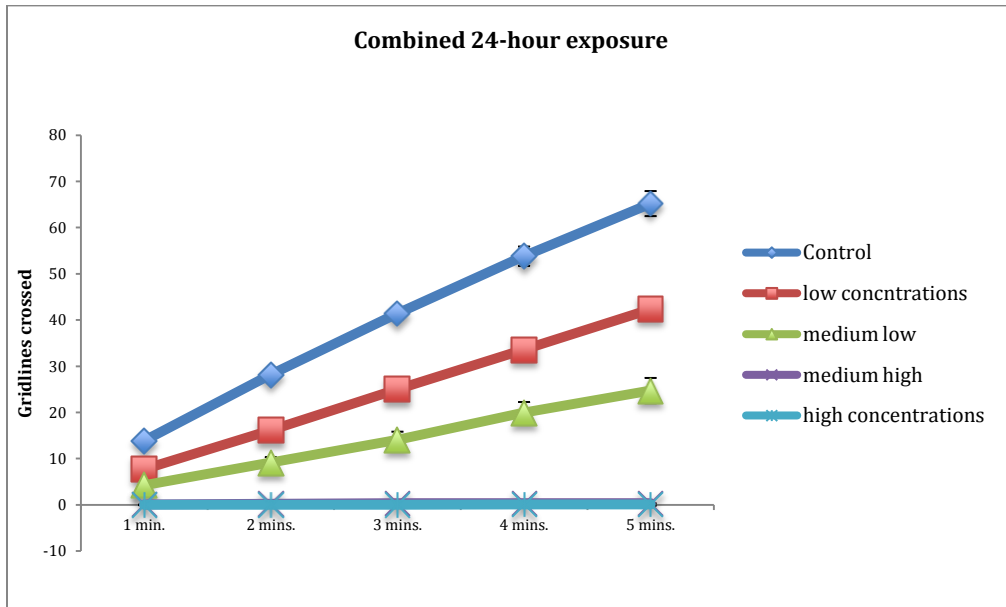


Figure 7.

Number of gridlines crossed cumulatively over 5-minutes after 24-hour exposure in terms of concentration for combined alcohol and nicotine.



Simple effects of exposure

Analysis of the simple effects for exposure time revealed a significant difference in the mean of gridlines crossed for the low concentration of nicotine, $F(1,364)=12.8$, $p<.001$. Similarly, the mean of gridlines crossed for ethanol revealed significant differences in terms of exposure for the low concentration, $F(1,364)=21.8$, $p<.001$. Additionally, the medium-low and medium-high concentrations for ethanol also showed significant differences, $F(1,364)=48.5$, $p<.001$ and $F(1,364)=17.1$, $p<.001$ respectively. Interestingly, the combined alcohol and nicotine group only showed mean differences in terms of exposure for the medium-low concentration, $F(1,364)=9.0$, $p<.005$.

Atypical behaviours

The total atypical withdrawal behaviours observed over a 5-minute period were analyzed using a MANOVA. There was a significant interaction between type, concentration and exposure on the number of observed atypical behaviours, $\Lambda=.813$, $F(36, 1579) = 2.117$, $p < .001$.

Separate univariate ANOVAs on the various atypical behaviours revealed a significant 3-way type by concentration by exposure interaction effect on all behaviours (headbop, $F(6,364)=2.2$, $p < .05$; corkscrew, $F(6,364)=2.8$, $p < .05$; tailtwist, $F(6,364)=3.0$, $p < .01$; squirming, $F(6,364)=4.4$, $p < .001$; clinging, $F(6,364)=2.7$, $p < .05$), with the exception of the headswing, $F(6, 364)=.665$, $p=.618$.

After 1-hour exposure, headbop, tailtwist, and squirming behaviours are significantly increased ($p < .05$) in the combined group compared to nicotine, ethanol and control groups in the low (Figure 8) and medium-low concentrations. After 24-hour exposure, headbop, tailtwist and squirming behaviours are significantly increased ($p < .05$) in the combined group compared to only the nicotine and control groups in the low (Figure 9) and medium-low concentrations. No difference is seen between the combined and ethanol groups at this point.

Figure 8.
 The number of observed atypical behaviours in the lowest concentration over a 5-minute period after 1-hour exposure.

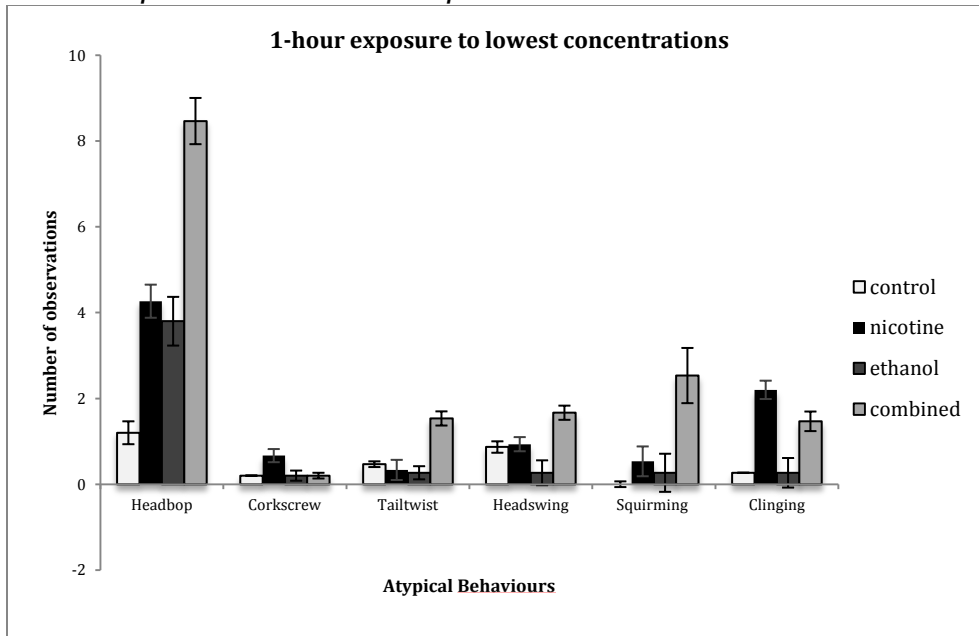
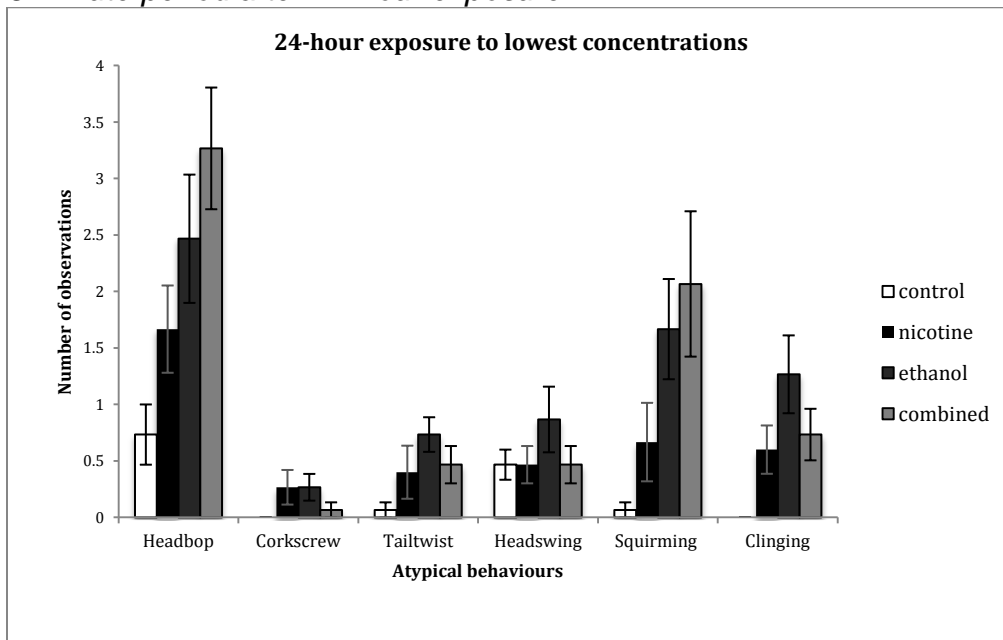


Figure 9.
 The number of observed atypical behaviours in the lowest concentration over a 5-minute period after 24-hour exposure.



In contrast, the general trend in the remaining higher concentrations is a significant decrease ($p < .05$) in headbop, clinging and squirming behaviours in the combined group compared to the ethanol and nicotine groups after 1-hour exposure. After 24-hour exposure to higher concentrations there was no significant difference between any of the drug groups in terms of atypical behaviours.

Discussion

One of the main challenges in studying alcohol and nicotine co-usage is in understanding how they interact (NIAAA, 2000). Since both drugs work on some of the same mechanisms in the brain, it has proven rather difficult for researchers to tease apart the individual and combined effects of these drugs (NIAAA, 2000). Based on the results of this study, the interaction is not as clear-cut as expected, but is rather complex with various ways of interacting dependent on the various factors involved (concentration and exposure time).

As expected both behavioural paradigms support evidence of a withdrawal phenomenon in planaria with significant differences between drug groups and the control group. Since drug groups, (with the exception of the lowest .01% alcohol group) had significantly ($p < .05$) decreased locomotion (pLMV) compared to controls, this study allowed for the application of the withdrawal phenomenon described in cocaine and opioids to alcohol and nicotine. The increase in atypical behaviour observations in the drug groups compared to controls also provides supportive evidence of this withdrawal phenomenon.

The main hypothesis proposed a significant difference in both behavioural paradigms between the single and poly-drug groups, with a synergistic effect occurring between the alcohol and nicotine. Results effectively showed significant differences in pLMV between the single and poly drugs groups with the locomotion of the combined group being generally increased in comparison to nicotine, but decreased in comparison to ethanol. Planaria locomotion was therefore drastically decreased in the nicotine compared to ethanol groups. This single drug versus the poly drug group distinction was greater in the acute 1-hour exposure, but still visible in the medium-low concentration of the 24-hour exposure. The drastic difference in locomotion between nicotine and alcohol was significantly increased in the 24-hour compared to the 1-hour exposure time.

Essentially, it appears that in the poly drug group, nicotine has the overall greater effect, but the alcohol lessens its effect. Thus the same high concentration of nicotine is not as potent when combined with ethanol in the acute 1-hour exposure, and this appears to be independent of the concentration until the highest concentration is reached. The effect of alcohol on the potency of nicotine appears reduced however as the effects become long-term in the 24-hour exposure. It can be hypothesized that neuroadaptations of the planarian nervous system in the regulation of ethanol have occurred, lessening its debilitating effects from the acute (1-hour) to long-term (24-hour) phases.

As previously mentioned, the atypical behaviours in planaria each represent its own specific receptor-binding site and are therefore critical in providing knowledge of the receptors involved in the drug mechanism. Their

specificity was initially determined when Venturini and colleagues, discovered screw-like (corkscrew) behaviours corresponded to dopamine D1 and C-like curling was found to correspond to dopamine D2 receptors (Venturini et. al., 1989). Since then, the screw-like behaviour has also been linked to the k-opioid receptors (Raffa et al., 2003), while C-like curling was found to correspond to glutamate receptors (Rawls, 2011). The differences in atypical behaviours observed between single and poly drug groups support the idea that different receptors are affected and have more or less activity in poly drug versus single drug groups. This difference in receptors provides the support and reasoning to imply that poly-drug use has different mechanisms in the brain than single drug use. Thus, we can imply that the mechanism or pathways for the action of the combined nicotine and ethanol is somewhat different than that of sole ethanol or nicotine.

The significant differences in exposure time varied dependent on the drug type. Long-term (24-hour) exposure caused significant decreases in locomotion in the nicotine-treated planarian, which may be attributed to significant damage to the system. However, in ethanol-treated planaria the nervous system appeared to show a form of neuroadaptation by developing a tolerance, which allowed for increased locomotion between the acute 1-hour and long-term 24-hour exposures.

Implications and future directions

Overall, the results for the study showed that there is indeed a significant difference between the effects of single drug and poly-drugs in the planaria,

specifically in regards to the co-use of alcohol and nicotine. With alcohol and nicotine co-use being so rampant and the numerous detrimental impacts globally that it holds on society, the elucidation of the mechanism of the interaction would provide the key to why their co-occurrence is so common.

The difference in atypical behaviours is a small clue in elucidating the mechanisms of this interaction. Currently however, these behaviours have only been quantified and described as a withdrawal phenomenon (Raffa and Valdez, 2005). Unfortunately, at this point in 2015, the corkscrew and C-like curling behaviours are the only behaviours in which the corresponding receptors have been determined. Thus, further research is necessary and the key to determining the corresponding receptors for the atypical behaviours that were found to show significant differences in this study. By figuring out the behaviour to receptor relationship, it will be easy to classify and have more insight into the differing and overlapping mechanisms behind the complex alcohol and nicotine relationship. Essentially, by figuring out the what, where and why behind the mechanisms between alcohol and nicotine complex, we can then determine how to better treat these kind of poly-drug addictions.

One of the biggest debates in addiction treatment facilities is whether or not treatment strategies for single drug addictions should differ if a person is addicted to more than one drug simultaneously. The results in this study provide evidence to support the idea that the co-abuse of alcohol and nicotine should have different treatments than those offered for single drug addictions to either substance.

In a broader sense, this study provides a small stepping-stone into poly-drug research altogether. Since real life scenarios have greater instances of poly drug-use it is important that the interactions between drug addiction mechanisms in the brain are understood in order to provide adequate addiction treatments. Further research into not only the alcohol and nicotine relationship, but also other commonly co-used drugs such as alcohol and MDMA, alcohol and cocaine, or alcohol and marijuana warrant further insight.

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